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Complement Activation and Perinatal Outcomes in African American Women

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Complement Activation and Perinatal Outcomes in African American Women

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An abstract of
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Abstract

Complement Activation and Perinatal Outcomes in African American Women
By Alexis Brennae Dunn

**Background:** Complement activation during pregnancy is essential for processes related to maternal-fetal immune tolerance and the immune response to infection. However, recent studies in predominantly white populations have shown that elevated levels of complement factors C3a and Bb during early pregnancy are associated with an increased risk for preterm birth (PTB). Bacteria are potent activators of complement, however the relationship between the microbiome and complement activation has not been explored in a pregnant population at high risk for PTB.

**Purpose:** This study explored the relationship between the vaginal microbiome, markers of complement activation, PTB associated clinical measures, and behavioral risk factors linked to infection and PTB.

**Sample and Design:** A prospective longitudinal study of 144 pregnant AA women enrolled in a larger study investigating biobehavioral determinants of the microbiome and the risk of PTB in AA women (1R01NR014800) were followed in this dissertation study. Women completed sociodemographic questionnaires and provided information about health behaviors and infection history. Blood and vaginal microbiome samples were collected between 8-14 weeks for evaluation of the vaginal microbiome and C3a/Bb levels. Medical record abstraction was completed to obtain data on clinical variables of interest (twenty-week cervical length and gestational age at delivery). Correlational and linear regression analysis were conducted to explore relationships between the select variables.

**Results:** The vaginal microbiome composition clustered into five distinct community state types (CSTs): (1) CST 1 *Lactobacillus*; (2) CST 2 *Prevotella/Bacteroides*; (3) CST 3 *Snethia/Gardnerella*; (4) CST 4 *Lactobacillus iners*; (5) CST 5 *Shuttleworthia*. Linear regression analyses concluded that neither the vaginal microbiome CSTs nor reproductive tract infection were associated with early pregnancy C3a or Bb levels. Similarly, neither C3a or Bb were significant predictors of the 18-20 week cervical length measurement or the gestational age at delivery. Age was significantly associated with increased C3a levels and a longer gestational age at delivery.

**Conclusions:** The vaginal microbiome CSTs and reproductive tract infection are not associated with C3a/Bb levels. Additionally, C3a/Bb levels are not associated with the clinical outcomes of interest. More studies are needed to identify factors that are associated with early pregnancy complement system activation in AA women.

# Complement Activation and Perinatal Outcomes in African American Women

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# Chapter 1: Introduction

## Significance of the Problem

Preterm Birth (PTB, birth <37 weeks gestation) is the most common adverse pregnancy outcome affecting nearly 500,000 infants annually and is the most significant contributor to infant mortality in the United States (CDC, 2014). In addition to the emotional and economic toll on families, PTB poses a significant burden on the US health care system with healthcare costs in excess of 26 billion dollars each year (MarchOfDimes, 2015). Spontaneous PTB, defined as birth resulting from preterm labor or preterm premature rupture of membranes, occurs in 70% of preterm deliveries (Lockwood, 2014). The risk for PTB, however, is not evenly distributed within the US population. Racial disparities persist among African American (AA) women who are 1.5 times as likely to have PTB (16.8%) as Caucasian (10.8%) or Asian (10.4%) women (Dimes, 2014a; Hamilton, Hoyert, Martin, Strobino, & Guyer, 2013). There are also racial disparities in the occurrence of inflammation-associated PTB, with AA women more likely to be affected (Lockwood, 2014; Hitti, 2007). Even more concerning is that the AA-Caucasian racial disparity in PTB is especially great for early PTB < 34 weeks (6.1% vs. 2.9%), the category of PTB for which the associated infant and long-term morbidity and mortality is the greatest (Dimes, 2014b). Although >80% of AA women go on to deliver healthy babies at term, a better understanding of biobehavioral risk factors for PTB within the population of pregnant AA women may provide additional insight into mechanisms and potential targets for intervention to eliminate this stubbornly persistent racial disparity in PTB (Menon, Dunlop, Kramer, Fortunato, & Hogue, 2011).

# Etiology of Preterm Birth

## Infection and Inflammation

The etiology of spontaneous PTB is multifactorial and thought to involve complex multifactorial pathways of both maternal and fetal origin. The exact pathophysiologic pathway to PTB is not fully understood, however intrauterine infection and inflammation have been identified as definitive risk factors in the pathogenesis of PTB. (Goncalves, Chaiworapongsa, & Romero, 2002; Holst, Mattsby-Baltzer, Wennerholm, Hagberg, & Jacobsson, 2005; Vogel, Thorsen, Curry, Sandager, & Uldbjerg, 2005). Several PTB studies have isolated pathogenic microbes from the chorioamnion and amniotic fluid of women with PTB, (DiGiulio, 2012; Marconi, de Andrade Ramos, Peracoli, Donders, & da Silva, 2011) suggesting that infection driven inflammatory events at the maternal fetal interface may be predictive of PTB. Exaggerated production and/or poor regulation of inflammatory mediators can have deleterious effects on various blood vessels and tissues, resulting in a host of pregnancy complications including PTB (Goldenberg, Hauth, & Andrews, 2000; Hayashi, Ueda, Ohkura, & Inaba, 2005; Holst et al., 2005; Romero, Gotsch, Pineles, & Kusanovic, 2007; Vogel et al., 2005).

There are four recognized pathophysiologic pathways to PTB: (1) premature activation of the maternal and/or fetal HPA axis; (2) pathological uterine distention; (3) abruption; (4) exaggerated inflammatory response/infection (Lockwood, 2014). Of these exaggerated inflammation is the effector pathway considered to account for AA's excess rates of PTB (Hitti J, 2007), as supported by elevated rates of early PTB < 34 weeks' and

rates of PTB preceded by PPROM (preterm premature rupture of membranes prior to the start of labor and also occurring <37 weeks gestational age), both more likely to occur in the setting of systemic and/or intrauterine infection. Intrauterine infection caused by the invasion of bacteria into the uterine cavity is the leading cause of infection-associated PTB (Goldenburg, 2008; Romero et al., 2006; Vogel et al., 2005). The pathway to microbial invasion of the amniotic cavity is thought to result primarily from ascending microbes from the vagina and cervix, however other pathways have been proposed including transplacental infection, retrograde exposure from the peritoneal cavity through the fallopian tube, or introduction during invasive procedures such as amniocentesis (Romero et al., 2001). Recent evidence supports hematogenous transfer of oral microbes to the placenta as well (Aagaard et al., 2014).

**The Vaginal Microbiome**

The most common pathway to intrauterine infection is thought to involve the ascension of pathogenic microbes from the vagina and cervix into the uterus which stimulate inflammatory mechanisms located in the uterus, cervix, and amniotic membranes (Bastek, Gomez, & Elovitz, 2011; Takeuchi & Akira, 2010). Vaginal infections such as bacterial vaginosis (BV) are highly associated with PTB (Culhane & Goldenberg, 2011; Denney & Culhane, 2009), and occur more frequently among AA women (Control, 2010; Kramer & Hogue, 2009; Menon et al., 2011). Pathogenic microbes associated with bacterial vaginosis known to be highly associated with PTB, such as *Gardnerella Vaginalis* and *Prevotella* disturb protective microbial species (e.g. *Lactobacillus*) resulting in infection (Culhane & Goldenberg, 2011; Denney & Culhane, 2009), particularly among AA women who are more commonly lactobacillus deficient

(Denney & Culhane, 2009; Zhou et al., 2007). AA women also have an increased incidence of sexually transmitted infections which are associated with chronic inflammation and increased risk of adverse pregnancy outcomes compared to other ethnicities of US women (Christian, 2012; Hogben & Leichliter, 2008; Romero et al., 2007). Despite the relationship between infection and PTB risk, randomized controlled trials directly targeting the elimination of infection during pregnancy have shown limited efficacy in reducing the risk of PTB (Lynch, Wagner, Deterding, et al., 2016; Lynch, Wagner, Giclas, et al., 2016; Slattery & Morrison, 2002), suggesting that there may be other more complex mechanisms involved in the infection associated pathway to PTB, particularly among AA women.

**The Complement System**

The complement pathway ("complement") is a key mediator of the inflammatory response involved in the first line innate defense against pathogens. The pathway is composed of more than 30 soluble and membrane bound proteins which in turn initiate a series of enzymatic reactions that function to irreversibly bind to pathogens, eliminate immune complexes, and facilitate removal of products following tissue injury (Markiewski & Lambris, 2007; Sjoberg, Trouw, & Blom, 2009; Walport, 2001). The Complement Activation Pathway actually involves three pathways as described by Lynch and colleagues (Lynch et al., 2011); these pathways are the classical, lectin, and alternative pathways, which are triggered by various initiating components (e.g. immune complexes, bacterial carbohydrates, bacteria) for each pathway (Holers, 2008; Markiewski & Lambris, 2007). Regardless of the initiating component, all three pathways lead to enzymatic cleavage of complement component C3 by a pathway specific C3

convertase (enzyme) into fragments C3a and C3b. C3a is a cleavage product that functions as an anaphylatoxin, which recruits and activates inflammatory cells, amplifies local inflammation, and affects vascular permeability and smooth muscle contractility (Sjoberg et al., 2009; Walport, 2001). Bb is involved in the alternative pathway of complement activation and assists in the additional creation of C3 convertase (Vaisbuch et al., 2009). C3b opsonizes pathogens and initiates the activation of downstream complement proteins that form the membrane attack complex (C5-C9), leading to pathogen cell lysis and death. (Lynch et al., 2011; Tegla et al., 2011).

Complement is also activated to assist in vascular remodeling and removal of cellular debris during implantation of the fertilized egg, essential steps in a healthy pregnancy (Bulla, Bossi, & Tedesco, 2012; Lynch et al., 2012; Lynch et al., 2011). Specifically, in early pregnancy, complement is activated to eliminate infectious agents at the fetal-maternal interface and to enhance clearance of cellular debris (ex: apoptotic cells, subcellular membranes, and damage associated proteins), released during vascular remodeling of the maternal endometrium during implantation and placental development (Bulla et al., 2012; Lynch et al., 2012; Lynch et al., 2011; Sjoberg et al., 2009). However, as described below, a host of pregnancy complications have been associated with dysregulated complement system activation including spontaneous miscarriage (Caucheteux, Kanellopoulos-Langevin, & Ojcius, 2003; Girardi & Salmon, 2003), preeclampsia (Soto et al., 2010), and fetal death (Richani et al., 2005).

The complement system has a variety of triggers for activation, however dysregulation of the system may result from factors that promote excess activation and/or poor regulation, thereby promoting a heightened inflammatory state. Although the

complement system is necessary for normal physiologic function, recent studies have identified elevated 1st trimester complement activation fragment C3a as significant predictor of spontaneous PTB and preterm premature rupture of membranes, one in mice (Gonzalez, Franzke, Yang, Romero, & Girardi, 2011; Lynch et al., 2011). An earlier study of 1st trimester complement fragment Bb also reported that women with levels of complement fragment Bb in the top quartile were 4.7 times as likely to have a spontaneous PTB less than 34 weeks gestation compared to women in the lower quartiles (Lynch et al., 2008). A limitation of the human studies includes their racial homogeneity: only 7% of the women in the study of C3a and 5% of women in the study of Bb were African American—the group at greatest risk of PTB due to an infectious/inflammatory process (Culhane & Goldenberg, 2011). Deposits of complement activation products have also been found on reproductive tissues including the placenta, cervix, and decidual spiral arteries (Girardi, Bulla, Salmon, & Tedesco, 2006; Gonzalez et al., 2011), highlighting the potential for dysregulation of complement system to promote dysfunction in the reproductive tissues and lead to adverse pregnancy outcomes.

## Purpose of the Study

Current risk factors explaining the racial disparities in PTB are lacking, and less than half the AA/Caucasian disparity is explained via current risk factors related to socioeconomic factors and health behaviors (Dunlop, Kramer, Hogue, Menon, & Ramakrishan, 2011; Hogue, Menon, Dunlop, & Kramer, 2011; Kramer, Hogue, Dunlop, & Menon, 2011; Marret et al., 2007; McGrady, Sung, Rowley, & Hogue, 1992). The remaining factors underlying the high AA rate of PTB are largely unknown; however inflammatory pathways triggered via various health conditions, such as infection, are key

risk factors consistently correlated with the development of adverse pregnancy outcomes, particularly among AA women (Menon et al., 2011). Thus, the identification of factors that promote dysregulation of inflammation via pathways directly associated with intrauterine infection may improve the understanding of the excess occurrence of PTB among AA women.

The purpose of this study is to investigate the relationship between: (1) the composition of the vaginal microbiome and the presence of reproductive tract infection with complement activation fragments C3a and Bb levels; (2) C3a and Bb levels and select clinical measures (cervical length and gestational age at delivery), and (3) C3a and Bb levels for various biobehavioral risk factors associated with PTB (e.g. vaginal hygiene practices, sexual practices, substance use) among AA women, the women most at risk for PTB. Figure 1 outlines the timeline for collection of each variable of interest. The study includes 144 pregnant AA women who were enrolled into a larger study investigating biobehavioral determinants of the microbiome and the risk of PTB in AA women (1R01NR014800), and expands upon the sample and data analyses to address the following specific aims (A), research questions (RQ), and hypotheses (H):

***Specific Aim 1:*** Explore the relationship between the vaginal microbiome and reproductive tract infection and complement activation fragment C3a and Bb levels in AA women during early pregnancy (8-14wks).

RQ1.1: Does the composition of the vaginal microbiome and/or the presence of genital tract infection associate with levels of C3a/Bb?

H1.1: A vaginal microbiome composition with a higher prevalence of pathogenic bacteria will be associated with higher levels of C3a/Bb

H1.2: Reproductive tract infection prior to 20 weeks gestation will be associated with higher levels of C3a and Bb.

***Specific Aim 2:*** Investigate the relationship between complement activation fragment (C3a/Bb) levels in AA women during early pregnancy (8-14 weeks) and PTB associated clinical measures (20 week cervical length, gestational age at delivery).

H1: Levels of C3a/Bb will be inversely associated with cervical length at 20 weeks and/or gestational age at delivery.

***Exploratory Aim***: Do particular modifiable biobehavioral risk factors linked to preterm birth and AA race (e.g., vaginal hygiene practices, sexual practices, substance use) independently or interactively impact the relationships between the vaginal microbiome and complement levels (C3a/Bb) and/or complement levels (C3a/Bb) and PTB-associated clinical measures?

**Theoretical Framework**

In Figure 2, we present a conceptual model outlining a proposed complement activation pathway to PTB. The model and hypothesis are further described in Chapter 2. We posit that the pathogenic microbes, as determined by the composition of the vaginal microbiome or the presence of reproductive tract infection, activates complement pathways and potentially increases complement activation fragments C3a and Bb in the maternal vasculature and reproductive tissues. Women who have a healthy vaginal microbiome are colonized with bacteria that prevent the development of infection via the

production of bacteriostatic compounds, like lactic acid which prevent the growth of pathogenic microbes (Witkin, 2014). Women who are deficient in protective species of bacteria are more prone to colonization by pathogenic microbes. The persistent presence of pathogens in the vagina activate local innate immune mechanisms. However, when local innate mechanisms fail and pathogens continue to invade the cervicovaginal mucosa then systemic components such as complement are amplified to prevent further spread of the pathogen (Witkin, 2014).

Deposits of complement activation products have been found on various intrauterine tissues including the placenta, cervix, and decidual spiral arteries (Gonzalez et al., 2011). Complement dysregulation (defined as excess activation or poor regulation) promotes not only destruction of pathogens, but also can initiate damage to self tissues (Girardi et al., 2006). Activation of the complement pathway promotes changes in vascular permeability as well as the activities of other inflammatory cellular populations such as macrophages, which further enhance inflammation and the production of inflammatory mediators that break down collagen, stimulate uterine contractions, facilitate cervical ripening, and promote decidual activation, thereby increasing the risk for PTB (as measured by decreased length of gestation at delivery and/or shortened cervical length). We will also explore the independent and interactive effect of previously identified biobehavioral risk factors linked to preterm birth and AA race (sexual practices, vaginal hygiene practices, substance use) on the relationships between the vaginal microbiome, C3a/Bb levels, and PTB-associated clinical measures.

**Research Design and Methods**

A socioeconomically diverse cohort of 144 pregnant AA women were followed prospectively in this dissertation study to explore the relationships between (1) the composition of the vaginal microbiome and the presence of reproductive tract infection with complement activation fragments C3a and Bb levels; (2) C3a and Bb levels and select clinical measures (cervical length and gestational age at delivery), and (3) C3a and Bb levels for various biobehavioral risk factors associated with PTB (e.g. vaginal hygiene practices, sexual practices, substance use) Data were leveraged from a currently active, 5-year longitudinal study (the "Parent Study") (1R01NR014800) focused on the contribution of the microbiome and biobehavioral risk factors to preterm birth in AA women. Utilizing data collected during direct contact as part of the Parent Study at 8-14 weeks of pregnancy and review of the medical records post-delivery, we collected additional information on: 1$^{st}$ trimester C3a/Bb levels and 2$^{nd}$ trimester clinical measure of cervical length: these variables were evaluated in light of participant self-report of biobehavioral risk factors related to infection and inflammation including: vaginal hygiene practices (e.g. douching, use of vaginal products), and sexual practices (e.g. coital frequency, number of partners) in the preceding month.

*Setting and Sample Recruitment:* Pregnant AA women were recruited from those presenting to prenatal care clinics of Emory University Midtown (Emory Midtown, a private hospital) and Grady Memorial Hospital (Grady Hospital, a public one primarily serving indigent patients), each of which provide prenatal care to ~ 2,000 women annually. Given that socioeconomic status is a determinant of many health behaviors, the

diversity across these hospitals provided a diverse group of women in which to explore biobehavioral pathways associated with complement activation.

*Inclusion criteria* included: 1) AA race (via self-report). By limiting participation to AAs, this study focused specifically on the identification of intra-race risk factors for complement activation. 2) Singleton pregnancy between 8-14 weeks gestation as verified by clinical record. 3) Able to comprehend written and spoken English. 4) Age 18-35 years. 5) No chronic medical conditions or chronic medications as these may impact inflammatory responses. Post-enrollment exclusion criteria included: 1) Fetal death prior to labor; 2) Congenital anomalies (verified by record).

**Study Protocol and Measures**: Women who presented for their first trimester prenatal visit were asked if they were interested in learning about the Parent Study the "Microbiome and Biobehavioral Risk Factors for Preterm Birth in AA Women." Women who indicated a willingness to participate in the Parent Study provided Informed Consent and were enrolled into the study. The parent study protocol included a series of demographic, stress, dietary, and behavioral questionnaires related to vaginal hygiene practices and sexual partners and behaviors in the last month. Next participants were given verbal and pictorial instruction directing them how to obtain self-collection of rectal, vaginal, and oral swabs. Participants provided these swabs for later analysis of their microbiome at each site; however, the analysis of the vaginal swabs was the focus of this dissertation study. The samples were immediately handed to the research coordinator (waiting outside the room) and stored in 1 mL of Amies transport medium (Copan) and frozen upright on dry ice until transported to the lab, where they were stored at −80 °C until DNA extraction. The women were then accompanied to their prenatal blood draw,

and an extra sample of blood (30ccs) was drawn for later measurement of immune and inflammatory variables. Women were compensated $30 for their participation in the study. The dissertation study was approved under the parent study general IRB-approved investigations of "inflammatory markers" included in the informed consent, such that a separate consent for the measurement of complement markers was not needed. The sample selected for the dissertation study was chosen from a larger group of 184 women actively enrolled in the parent study who had an estimated date of delivery prior to the end of March 2016, and for whom 16s microbiome sequencing data would be available. Data and samples collected from a subset of 144 women who also had plasma available for analysis of complement levels were used to achieve the Aims identified in this dissertation study.

In Table 1, we describe the study variables and corresponding instruments that were collected in the Parent Study (**gray**) and dissertation study (**orange**). The parent study protocol has been included verbatim as written in the parent grant and placed in quotations for citation purposes. No additional meetings occurred with the women as part of the study and no additional compensation was issued, outside of the $30 provided by the parent study protocol.

***Table 1: Biological and Questionnaire Data Collection Instruments: Parent Study (Grey)/ Dissertation Study (Green)***

| **Items Collected** | **Type of Assay/Analysis** | **Description** |
|---|---|---|
| **Time Point 1: 8-14 weeks Gestation (Direct Participant Contact)** | | |
| Vaginal Swab | Vaginal Microbiome 16S rRNA sequencing | As described directly from the parent study protocol: "DNA were extracted from swab samples using the MoBio isolation Kit in line with the HMP Standard Operating Protocol. Microbial diversity will be characterized by DNA sequence variation of the 16S rRNA gene. Each sample DNA will be amplified in duplicate, to control for variation due to random PCR amplification artifacts. We will amplify the V4 region of the bacterial 16S genes using the primers and methods described by Caparoso (Caporaso et al., 2011) and |

| | | |
|---|---|---|
| | | sequence oligonucleotides using PCR primers containing multiplex tags that allow 96 samples per lane. Pooled amplicons will be sequenced on the Illumina MiSeq instrument using reversible terminator chain extension for 250 cycles from each side of the 16S DNA insert. For each run, we expect more than 10 million high quality paired reads (Q score > 30 at each base) for an average number of reads of at least 50,000 per sample, after control DNA removal. Metadata on each sample will be stored in a local database compliant with the MIMS (Minimal Information about a Metagenome Sequence) ontology. Processing of Sequence data and assignment to Operational Taxonomic Units (OTUs): Data processing, including demultiplexing, QC filtering, contamination and sample mislabeling data checks, OTU representation, taxonomy assignment via a reference database,(Caporaso et al., 2010; Wang, Garrity, Tiedje, & Cole, 2007) and phylogeny and diversity analysis (C. A. Lozupone, Hamady, Kelley, & Knight, 2007; C. Lozupone, Hamady, & Knight, 2006; C. Lozupone & Knight, 2005) will be accomplished using the QIIME75 pipeline and custom in-house software. We will compare the overall Shannon species diversity. Loss of taxonomic diversity in general is an indicator of disease state in many ecological systems. We will validate key findings using real time PCR assays designed for specific OTUs". |
| Sociodemographic Questionnaire | Questionnaire | "Self-report and prenatal record review to obtain family size and household income, age, years of education, marital status, and insurance status. Income will be determined by administrative review for government assistance at the clinic or hospital. All participants are AA based upon self-report." |
| Health Survey | Questionnaire | "Questions to ascertain, within the last month, diagnoses (including infections), medications (including antibiotics), sexual encounters (type of intercourse, use of condoms, number of partners), hygiene self-care practices (douching, feminine sprays/wipes), substance use (tobacco, alcohol, drugs)." |
| **Time Point 2: Delivery (medical record abstraction)** Medical record abstraction will be completed utilizing a standardized chart abstraction tool (Parker et al., 2011) to obtain the following data: | | |
| Gestational age | Chart Review | "Gestational age: Defined as the gestational week at the time of delivery, as determined by criteria at enrollment by LMP and/or early ultrasound. Gestational age at birth will be determined from the delivery record, based upon the date of delivery in relation to the EDC established by the 10-14 week prenatal visit." |
| Genital tract infection | Chart Review | "Clinical diagnoses of reproductive tract infections (BV, specific STIs) during pregnancy will be ascertained from the prenatal record, including clinical laboratory tests." |
| BMI | Chart Review | "Pre-pregnancy BMI will be calculated from measured height at the first prenatal visit and patient report of pre-pregnancy weight and categorized according to accepted definitions (obesity ≥ 30 kg/m$^2$, overweight 25-29.99 kg/m$^2$, healthy weight 18.5-24.99 kg/m$^2$, and underweight <18.5 kg/m$^2$)." |
| ***Biological and Questionnaire Data Collection Instrument added in the dissertation study*** | | |

| | | |
|---|---|---|
| Blood (Time Point 1) | Complement Activation Fragment C3a | **C3a was measured in the blood sample collected during the first visit of the Parent Study**. Plasma supernatant samples were batch analyzed for measurement of human C3a with a commercial ELISA kit (BD Biosciences, Opt EIA) according to manufacturer's instructions. **Bb was measured in the blood sample collected during the first visit of the Parent Study**. Plasma supernatant samples were batch analyzed for measurement of human Bb with a commercial ELISA kit (MicroVue Bb Plus Fragment EIA kit, Quidel Corporation, OH) according to manufacturer's instructions. |
| 20 Week Cervical Length (Time Point 2) | Chart Review | **20 week sonographic cervical length measurements, which are routinely collected on all pregnant women, were obtained during chart review post delivery**. |

## Proposed Data Analysis

The primary objective of this study was to explore biobehavioral risk factors for complement activation during early pregnancy among AA women. We analyzed data with the specific intent of establishing population parameters (e.g., effects sizes) for the variables being investigated. Data were analyzed using both descriptive and inferential test statistics. To identify distributions, outliers, and missing data, analysis first involved using measures of central tendency and dispersion to examine and compare the frequencies, means, and variability measures for select variables. Standard statistics and graphic software were used to summarize and visualize the characteristics of the data. Missing data were handled through standard methods for subjects with at least 85% completed data on a given variable. SPSS Missing Value Analysis procedures was used to evaluate missing data patterns, and sensitivity analysis was performed to evaluate the impact of missing data on results.

Our initial data exploration included: 1) determining the distributions of outcome measures and assessing whether data transformations were needed, 2) insuring that

underlying assumptions of statistical analyses were satisfied, 3) identifying potential co-linearity problems, 4) identifying potential outliers that would require further investigation, 5) confirmatory psychometrics for established behavioral measures. For categorical variables, we checked for sparse cells and regrouped categories if necessary. In building models, we checked linearity assumptions for continuous predictors and consider higher-order terms if needed. We also used simple bivariate correlations, t-tests, and chi-square as the test statistics. A sample size of 100 achieves 80% power to detect an R-Squared of 0.05 attributed to 3 independent variable(s) using an F-Test with a significance level (alpha) of 0.05000. The variables tested are adjusted for an additional 3 independent variable(s) with an R-Squared of 0.05. Analyses were performed using IBM SPSS (version 24) with level of alpha= .05 used to determine statistical significance.

***Aim 1/H1.1-1.2*:** **Explore the relationship between the vaginal microbiome, reproductive tract infection, and complement (C3a/Bb) levels in AA women during early pregnancy (8-14wks).** Multivariable linear regression analysis was used to characterize the relationship between the independent variables (vaginal microbiome and reproductive tract infection) and the dependent variable (C3a/Bb levels), controlling for select covariates. The vaginal microbiome composition was evaluated according to the relative abundance of select microbes and known bacterial pathogens (classified by community state type). Using the initial covariance matrix we evaluated multi-collinearity among the independent variables. Diagnostics (e.g., condition index, variance inflation factors and tolerance levels) were calculated to determine if multi-collinearity issues were present.

***Aim 2/H2.1*: Investigate the relationship between complement activation fragment C3a/Bb levels in AA women during early pregnancy (8-14 wks) and PTB associated clinical measures (20 week cervical length, gestational age at delivery).** Multivariable linear regression was used to characterize the relationship between C3a/Bb levels and PTB associated clinical measures. Multivariable linear regression methods and diagnostics as outlined in aim 1were used to build the models.

***Exploratory Aim*: Do particular modifiable biobehavioral risk factors linked to preterm birth and AA race (e.g., vaginal hygiene practices, sexual practices, substance use) independently or interactively impact the relationships between the vaginal microbiome and levels of C3a and/or levels of C3a and PTB-associated clinical measures?** A general linear model (GLM) would have been used to examine the relationships between predictor and criterion variables, along with a full factorial model to test for interactions and review estimated marginal means. However, the relationships explored in Aims 1 and 2 were not supported. As such, student t-tests were done to evaluate differences in the mean concentration of C3a and Bb for select health behaviors.

## Protection of Human Subjects

This study meets federal regulations defining minimal risk: "the probability and magnitude of harm or discomfort anticipated in the research are not greater in and of themselves than those ordinarily encountered in daily life or during the performance of routine physical or psychological examinations or tests [§46.102]." No intervention strategies were delivered or evaluated as a part of this research study, and the usual

standard of obstetrical care was not altered in any way for participants. Since the study involved research with a vulnerable population (pregnant women), special considerations [§45 CFR 46 (Subpart B)] were followed.

Recruitment & Informed Consent: Women were recruited from Emory OB/GYN prenatal clinics at Grady and Emory Midtown Hospitals during their $8^{th}$-$14^{th}$ week of pregnancy. This study involved a single consent procedure obtained at enrollment (8-14 weeks). To ensure safety and protection of patient privacy, the study overview, informed consent, and HIPPA consent were completed in a private room. Women were asked if they provided permission to store their data and biological samples without identifiers for future analyses to answer new research questions regarding health behaviors, the microbiome, and pregnancy complications. The dissertation study was approved under the parent study general IRB-approved investigations of "inflammatory markers" included in the informed consent, such that a separate consent for the measurement of complement markers was not needed.

Protection against risks*:* Answering questionnaires on sexual history, vaginal hygiene practices, and substance use may be stressful. We informed women that they could choose not to answer any question and could discontinue their participation in the study at any time. Any health problem identified was reported to the woman's primary care provider and the PI's of the Parent Study. ***Biologic Samples*:** Drawing blood via needle stick to the antecubital vein could be mildly uncomfortable and potentially could be a site for bruising or infection. The participants in this study did NOT undergo any additional blood draw as part of their participation, rather we obtained the blood samples with the same needle stick used to draw blood for the routine obstetrical laboratory

studies conducted at 8-14 weeks. Although no serious adverse events (e.g. hematoma arterial puncture, phlebitis) occurred, if any such occurrence had occurred, it would have been reported to the woman's primary care provider and Parent Study PIs. *Vaginal swabs* were collected as part of the Parent Study to extract microbial DNA for 16S rRNA sequencing for characterization of the vaginal microbiome. Participants performed self swabs in a manner that was consistent with existing protocols that report validity and safety in self collection during pregnancy. Participants were given verbal and pictorial instruction directing them in the process of swab self-collection. Universal precautions were used in the handling, processing, and disposing of biohazards.

Confidentiality: Breach of confidentiality was a potential risk; however, measures were implemented on several levels. First, all research investigators were trained in human research protections. Second, all data were protected from anyone who did not have a position in the study. To that end, all participants were assigned a study ID number upon entry into the study, and all data collected from then on was labeled only with the ID number. Only the parent study PI's and research coordinators had access to the password protected computer file matching ID numbers with names. Third, raw data were kept in locked filing cabinets. Fourth, all data collection was done on password-protected and encrypted computers with Redcap software, that allowed for transfer of data to the Emory server in a HIPAA-compliant manner. Fifth, the Emory server was also password protected, encrypted, and backed up to ensure data confidentiality and integrity. Finally, laboratory samples were kept in locked areas with ID numbers. Data and Safety Monitoring: Data and safety monitoring was conducted by the Parent Study PI's and appropriate reporting to the Emory IRB was completed if necessary. As this research

study does not involve an intervention, and does not constitute a clinical trial, a Data Safety Monitoring Committee was not required.

## Innovation of the Proposed Study

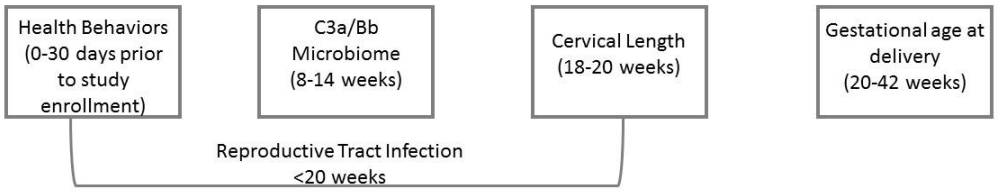
The proposed study was innovative in that it: 1) Explored an **innate** immune mechanism that has been understudied in the context of PTB -- complement activation – the dysregulation of which in early pregnancy may prove to be an underlying mechanism of preterm birth and whose elevation may someday lead to its use as a biomarker to predict its future occurrence. (2) Focused on the **population with the highest incidence of PTB-African American women**. (3) **Employed a design consistent with recommended frameworks for studying racial disparities**, which recognizes that the *first step* in studying disparities is to understand the interplay of risk factors *within* the disparate group (Cohen et al., 2012; Stark JL, 2001).

## Summary

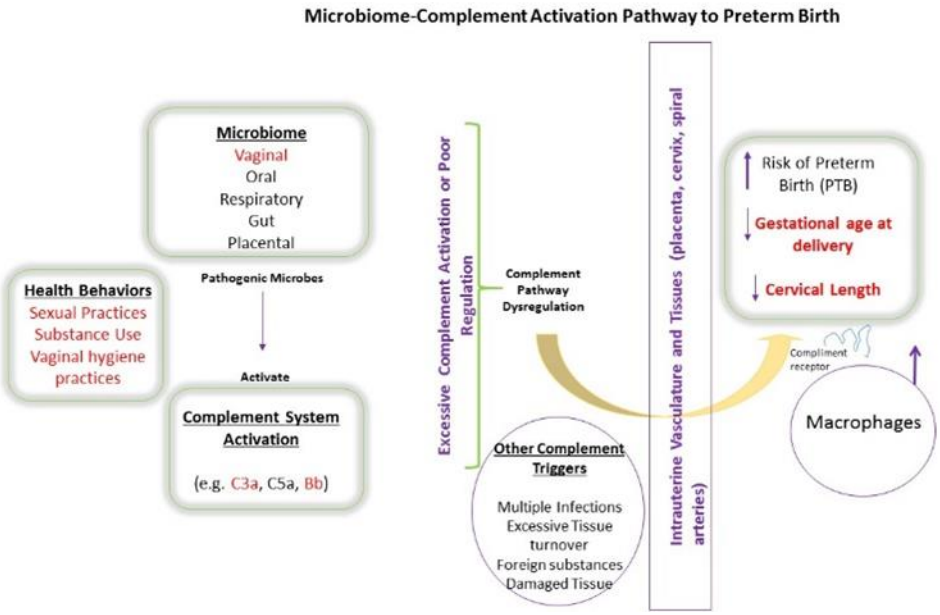
A prospective, longitudinal cohort study was conducted to explore the relationships between (1) the composition of the vaginal microbiome and the presence of reproductive tract infection with complement activation fragments C3a and Bb levels; (2) C3a and Bb levels and select clinical measures (cervical length and gestational age at delivery), and (3) C3a and Bb levels for various biobehavioral risk factors associated with PTB (e.g. vaginal hygiene practices, sexual practices, substance use) among African American women. Each specific aim has been analyzed and is presented in chapters 2-4 of this dissertation. Each chapter is prepared for submission to a peer-reviewed journal as

selected by the author. An integrative summary and analysis with implications for future research is outlined in Chapter 5.

## Figure 1




## Figure 2



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# Chapter 2: The Microbiome and Complement Activation: A Mechanistic Model for Preterm Birth


## Abstract

Preterm Birth (PTB, < 37 completed weeks' gestation) is one of the leading obstetrical problems in the United States affecting approximately 1 of every 9 births. Even more concerning are the persistent racial disparities in PTB with particularly high rates in African Americans. There are several recognized pathophysiologic pathways to PTB, including infection and/or exaggerated systemic or local inflammation. Intrauterine infection is a causal factor linked to PTB, thought to result most commonly from inflammatory processes triggered by microbial invasion of bacteria ascending from the vaginal microbiome. Trials to treat various infections have shown limited efficacy in reducing PTB risk, suggesting that other complex mechanisms, including those associated with inflammation, may be involved in the relationship between microbes, infection, and PTB.

A key mediator of the inflammatory response, and recently shown to be associated with PTB, is the complement system, an innate defense mechanism involved in both normal physiologic processes that occur during pregnancy implantation, as well as processes that promote the elimination of pathogenic microbes. The purpose of this paper is to present a mechanistic model of inflammation-associated PTB, which hypothesizes a relationship between the microbiome and dysregulation of the complement system. Exploring the relationships between the microbial environment and complement biomarkers may elucidate a potentially modifiable biological pathway to preterm birth.


Keywords: Preterm Birth, Microbiome, inflammation in pregnancy, complement system

Preterm birth (PTB), defined as birth <37 completed weeks' gestation, is one of the leading obstetrical problems in the United States (US), affecting nearly 500,000 – or 1 of every 9 – infants born annually (CDC, 2014). PTB is the greatest risk factor for infant death, contributing to 35% of all infant deaths and healthcare costs in excess of $26 billion annually (MarchOfDimes, 2015). Although PTB rates have declined in the general US population since 2007, the overall rate remains around 12% (CDC, 2014; Martin, Hamilton, Osterman, Curtin, & Matthews, 2015). Additionally, the incidence of PTB persists as a problem among ethnic and racial subgroups (Culhane & Goldenberg, 2011). One of the groups at the highest risk, African American (AA) women, are 1.5 times as likely to experience PTB and nearly 2 times as likely to have an early PTB (<32 weeks) compared to Caucasian women (Culhane & Goldenberg, 2011; Kramer, Hogue, Dunlop, & Menon, 2011; MarchOfDimes, 2014). Less than half the AA/Caucasian disparity is explained via current risk factors associated with PTB such as maternal age, history of infection, and substance use (Goldenberg, Culhane, & Iams, 2008; McGrady, Sung, Rowley, & Hogue, 1992). Identification of novel biobehavioral pathways to PTB may prove beneficial, particularly in groups with the greatest risk.

Although many mammalian species exhibit slight variations in gestational length, PTB appears to be primarily a human phenomenon (Phillips, Abbot, & Rokas, 2015). Identification of the underlying biological mechanisms of PTB is difficult as the complication is syndromic in nature and includes biological (e.g., infection, HPA axis activation, uterine over distention), behavioral (e.g., stress, substance abuse, anxiety), and epidemiologic (e.g., demographic, social, economic) contributing factors (Goldenberg, Culhane, & Iams, 2008; Kramer & Hogue, 2009). Of the many biological processes

implicated in the onset of PTB, intrauterine infection and/or inflammation are strongly associated with an increased risk for PTB (Goldenberg, Hauth, & Andrews, 2000; Goncalves, Chaiworapongsa, & Romero, 2002; Romero et al., 2006).

In this pathway to PTB, microbial invasion triggers inflammation, which is driven by the release of pro-inflammatory cytokines, prostaglandins, and matrix metalloproteinases (MMP) that promote cervical ripening and weakening of the amniotic membranes. This suggests that mechanisms that alter inflammatory events at the maternal fetal interface may be predictive of PTB (Holst, Mattsby-Baltzer, Wennerholm, Hagberg, & Jacobsson, 2005; Vogel, Thorsen, Curry, Sandager, & Uldbjerg, 2005). Despite this relationship, randomized control trials directly targeting known contributors of intrauterine infection, including the elimination of bacterial vaginosis (BV), sexually transmitted infections, and periodontal disease during pregnancy, have shown limited efficacy in reducing the risk of PTB (Lynch, Wagner, Deterding, et al., 2016; Lynch, Wagner, Giclas, et al., 2016). These findings suggest that there may be other more complex mechanisms involved in the infection associated pathway to PTB.

The inflammatory response to infection is mediated by the activities of several immune cell populations, including a group of blood proteins known as complement. The complement system is an innate immune mechanism composed of more than 30 blood proteins that initiate a series of enzymatic reactions that drive the initiation of the inflammatory response that ultimately results in elimination of a pathogen (Markiewski & Lambris, 2007; Sjoberg, Trouw, & Blom, 2009; Walport, 2001). Complement dysregulation, defined as excess activation or poor regulation, may promote an exaggerated inflammatory response and damage to self-tissues in the process (Girardi,

Bulla, Salmon, & Tedesco, 2006). The complement pathway has recently been associated with cervical remodeling, preterm premature rupture of membranes, and PTB (Gonzalez, Franzke, Yang, Romero, & Girardi, 2011; Lynch et al., 2011), suggesting that complement dysregulation in the intrauterine tissues and maternal vasculature may be predictive of PTB.

Although not previously linked together in the literature in regard to PTB, complement and the microbiome are linked in oral (Astafurov et al., 2014; Boackle, 1991; Hajishengallis & Lambris, 2012; Hajishengallis et al., 2011; Hajishengallis, Maekawa, Abe, Hajishengallis, & Lambris, 2015), gut (Lu, Knutson, Wishnok, Fox, & Tannenbaum, 2012; Yoshiya et al., 2011), skin (Chehoud et al., 2013), and nasopharynx (Domenech, Ramos-Sevillano, Garcia, Moscoso, & Yuste, 2013) health. Given the ability of the microbiome to influence the expression of complement in other situations, and the suspected associations of vaginal and even oral microbial dysbiosis as a risk for PTB, we summarize the current knowledge regarding the role of infection and inflammation in the context of PTB, with a specific focus on the role of the microbiome and the complement system. We conclude with a presentation of a mechanistic model for inflammation-associated PTB, which hypothesizes a relationship between the microbiome and dysregulation of the complement system. Identification of biological pathways of inflammation, particularly in high risk groups, may identify potential targets for intervention to eliminate this stubbornly persistent adverse pregnancy outcome and health disparity.

## Mechanisms of Preterm Birth: Infection and Inflammation

The study of PTB has involved the investigation of a variety of anatomical, endocrinological, immunological, and clinical events that affect both the mother and fetus. While many organ systems are involved in the labor process, changes within the intrauterine environment are directly involved in the labor and birth process. The accepted framework is that preterm parturition results from the physiologic activation of pathways resulting in increased uterine contractility, cervical ripening, amniotic membrane weakening, and decidual activation (Romero et al., 2006; Romero, Gotsch, Pineles, & Kusanovic, 2007). The mechanisms involved in initiating the process remain unclear, but one of the major focal areas of obstetrical research is the study of pathogenic processes highly associated with PTB, including infection and inflammation.

### Infection and Preterm Birth

Evidence suggesting a relationship between infection and preterm parturition is readily available: (1) Extrauterine infections such as pylenophritis, (Kaul et al., 1999; Wren, 1969) pneumonia, (Madinger, Greenspoon, & Ellrodt, 1989; Munn, Groome, Atterbury, Baker, & Hoff, 1999) and periodontal disease (Goepfert et al., 2004; Jeffcoat, Geurs, Reddy, Goldenberg, & Hauth, 2001; Offenbacher, 2004) are associated with PTB; (2) Several PTB studies have identified pathogenic microbes from the chorioamnion and amniotic fluid of women with PTB; (DiGiulio, 2012; Marconi, de Andrade Ramos, Peracoli, Donders, & da Silva, 2011); (3) Subclinical intrauterine infection is associated with PTB (Gomez et al., 1995); and (4) Animal studies show that intrauterine infection or systemic microbes stimulate preterm labor and birth (Gonzalez et al., 2011; Romero et al., 1988). In fact, intrauterine infection caused by the invasion of bacteria into the uterine

cavity is the leading cause of infection-associated PTB (Goldenberg, et al., 2008; Romero et al., 2006; Vogel et al., 2005). The pathway to microbial invasion of the amniotic cavity is thought to result primarily from ascending microbes from the vagina and cervix, however other pathways have been proposed including transplacental infection, retrograde exposure from the peritoneal cavity through the fallopian tube, or introduction during invasive procedures such as amniocentesis (Romero et al., 2001). Recent evidence supports hematogenous transfer of oral microbes to the placenta as well (Aagaard et al., 2014) .

Intrauterine infection accounts for 25-40% of preterm births (Goldenberg et al., 2000; Goncalves et al., 2002). The isolation of microorganisms from the amniotic cavity is considered an abnormal finding as the uterus is traditionally thought to be sterile. However microbial invasion of the amniotic cavity (MIAC) has been shown in women with preterm labor (PTL-regular uterine contractions with cervical change<37 weeks gestation), PTB (delivery <37 wks gestation), and preterm premature rupture of membranes (PPROM rupture of membranes <37 weeks gestation). Studies investigating the relationship between MIAC and PTL in women with and without intact membranes support differences in the prevalence of MIAC pending the patient clinical presentation, with greater microbial invasion being associated with actual preterm delivery and PPROM. The mean rate of positive amniotic fluid cultures in women with PTL and intact membranes is 12.8%, whereas with preterm labor and delivery with intact membranes it is 22%, and for women with PPROM is 32.4% (Goncalves et al., 2002). MIAC may occur in 51% of women who present with cervical shortening (Romero et al., 1992); a shortened cervix by sonography (cervical length <25mm) is a significant predictor of

preterm delivery (Iams et al., 1996; Moroz & Simhan, 2014). Common microorganisms isolated from women with intrauterine infection and PTB include *Mycoplasma*, *Streptococcus*, *Ureaplasma*, *Bacteroides*, and *Prevotella* species. (Hill, 1998). These findings suggest that microbial invasion and inflammation in the maternal reproductive tissues may be predictive of PTB.

## Inflammation and Preterm Birth

### Review of the inflammatory response to infection

The immune system protects the body from infection and invasion by microorganisms via a highly complex and organized system of various cell-based populations that interact via various effector mechanisms to eliminate pathogens (Mogensen, 2009). Macrophages are one of the first effector cells to be activated in the innate immune response as they are resident in the soft tissues. Macrophages and other cellular populations use a variety of pattern recognition receptors such as complement receptors (e.g., CR1, CR3, CR4), mannose, and toll-like receptors (TLR) to recognize molecular structures found on the surface of pathogens (Hargreaves & Medzhitov, 2005; Mogensen, 2009; Taylor et al., 2005). Upon exposure to bacterial antigens, a signal transduction cascade is triggered which results in the activation of NF-kappaB (NF-κβ) and other transcription factors that regulate the genes involved in the production of inflammatory cytokines; these cytokines then attract other cellular populations to the site of infection (Lawrence, 2009). There are many different types of cytokines that can be secreted, but interleukin 1 beta (IL-1 β), interleukin 6 (IL-6), interleukin 8 (IL-8), and interleukin 12 (IL-12) are heavily involved in the recruitment of different cellular population during the early stages of infection (Janeway & Medzhitov, 2002). TNF-alpha

(TNF-α) in turn drives the vasodilation of blood vessels and is heavily involved in endothelial leukocyte cellular activity (Bradley, 2008), which allows for the movement of fluid, plasma proteins, and white blood cells (WBC) into the tissue resulting in inflammation.

**Role of exaggerated inflammation in PTB**

There is strong evidence supporting the role of various inflammatory mediators in the etiology of PTB (Gibbs, Romero, Hillier, Eschenbach, & Sweet, 1992; Gomez et al., 1995; Keelan et al., 2003). The release of various inflammatory cytokines, such as IL-8, IL-1β, and TNF-α, along with microbial endotoxins, is thought to stimulate the production of other inflammatory mediators including prostaglandins and MMPs (Goldenberg et al., 2000; Keelan et al., 2003; Romero et al., 2007). IL-1β in fact was one of the first cytokines implicated in the onset of spontaneous preterm labor (Romero, Durum, et al., 1989) and is further supported by the finding that the human decidua produces IL-1β in response to bacteria (Romero, Wu, et al., 1989). IL-1β concentrations are increased in the amniotic fluid of women with preterm labor and infection, and IL-1β is thought to stimulate the production of prostaglandins and initiate intrauterine contractions (Marconi et al., 2011; Nadeau-Vallee et al., 2016). Similarly, TNF-α can stimulate prostaglandin production in the intrauterine tissues, and also in response to bacterial components; elevated TNF-α concentrations have also been shown in women with PPROM and intrauterine infection, and increased TNF-α has been identified as a mechanism for bacteria induced preterm birth (Hillier et al., 1993; Romero et al., 2007). Other cytokines including IL-6, IL-10, and IL-18 have also been implicated in the biologic pathway to PTB as well. Early and prolonged activation of these inflammatory

processes are thought to break down collagen, stimulate uterine contractions, facilitate cervical ripening, and promote decidual activation, resulting in incompetent cervix and amniotic membrane weakening; (Goldenberg et al., 2000; Romero et al., 2007); these are key risk factors in the development of PTB (Holst et al., 2005; Romero et al., 2007).

## The Microbiome as a Mechanism for Preterm Birth

The study of the human "microbiome" refers to the evaluation of the composition and metabolic potential of the ecologic community of microbes residing within the human body (Peterson et al., 2009). Evaluation of a variety of microbes found in different body sites have been made possible as a result of newer culture-independent DNA sequencing strategies using polymerase chain reaction (PCR methods), which have allowed for the identification of a significantly greater number and diversity of intrauterine microbes not typically found with culture dependent methods (Shendure & Ji, 2008). PCR analysis of amniotic fluid of women with PTB indicates a 30-50% higher prevalence of intrauterine infection and identifies a 1.5-3.5 times greater number of bacterial taxa as compared to studies using culture based methods (DiGiulio, 2012; Han, Shen, Chung, Buhimschi, & Buhimschi, 2009; Marconi et al., 2011). Specifically16S ribosomal rRNA gene based sequencing is used to characterize and compare bacterial communities, thereby allowing for a more detailed evaluation of the microbiota present (Eckburg et al., 2005; Lane et al., 1985). Using these newer methods to explore routes associated with intrauterine infection, such as the bacteria flora of the vagina, may be beneficial particularly in women at risk for PTB, and may explain a pathway to PTB in women who present without overt signs of clinical infection.

**The Vaginal Microbiome and PTB**

Although there are many proposed routes to intrauterine infection including hematogenous spread of microbes from the oral (Han et al., 2006; Solt, 2015), gut (Cani, Osto, Geurts, & Everard, 2012), and respiratory (Sandu, Folescu, Pop, & Motoc, 2013) communities, the primary pathway to intrauterine infection is thought to involve the ascension of pathogenic microbes from the vagina and cervix into the uterus (Bastek, Gomez, & Elovitz, 2011; Romero et al., 2001). The activities of the microbes present in the vagina form a complex ecosystem, collectively known as the vaginal microbime, which play a role in both normal physiologic function as well as infection. Protective bacterial species, such as lactobacillus (e.g., L. *crispatus*, L. *iners*, L. *jensenii*, L. *gasseri*), produce lactic acid and other bacteriostatic compounds that prevent the overgrowth of other, more pathogenic microbiota (Boskey, Cone, Whaley, & Moench, 2001; Kaewsrichan, Peeyananjarassri, & Kongprasertkit, 2006). When the vaginal equilibrium of lactobacilli are disturbed, either via replacement or due to the overgrowth of select anaerobes, vaginal infections such as BV are more likely to occur (Hummelen et al., 2010; McMillan et al., 2015).

The microbes that colonize epithelial surfaces of the vagina communicate with a wide variety of pattern recognition receptors in the uterus, cervix, and amniotic membranes (Bastek et al., 2011; Takeuchi & Akira, 2010) directly or through the release of products such as lipids, carbohydrates, proteins, or nucleic acids (Chu & Mazmanian, 2013). This communication facilitates the activation of various pro- and anti-inflammatory mechanisms to prevent the elimination of commensal bacteria and stimulate an aggressive inflammatory response to eliminate pathogenic bacteria, thereby

maintaining a healthy vaginal microenvironment (Chu & Mazmanian, 2013). A healthy vaginal microbiome plays a role in the prevention of several reproductive tract infections associated with preterm birth including BV, sexually transmitted infections, and urinary tract infections (Donders et al., 2000; Gupta et al., 1998; Martin et al., 1999; Wiesenfeld, Hillier, Krohn, Landers, & Sweet, 2003).

The vaginal tract is home to more than 50 non-pathogenic species of commensal flora (Cribby, Taylor, & Reid, 2008; Oakley, Fiedler, Marrazzo, & Fredricks, 2008). These flora vary widely among women due to host and environmental factors (Costello et al., 2009; Ravel et al., 2011). A recent study of the vaginal microbiome of 396 asymptomatic non-pregnant women from various ethnic groups identified 5 clusters of microbial flora, 4 of which were dominated by lactobacillus species, and varied significantly based on ethnicity (Ravel et al., 2011). Ethnic differences in the vaginal microenvironment is further supported by the fact that AA women are more likely to have vaginal microbiota that are not dominated by lactobacillus (Zhou et al., 2007). Pathogenic vaginal microbes known to be highly associated with PTB, such as *Gardnerella Vaginalis*, *Bacteroides*, *Mobiluncos*, and *Prevotella* disturb protective microbial species (e.g., *Lactobacillus*) resulting in vaginal infections, such as BV, the most common vaginal infection affecting women age 15-44 in the US (CDC, 2016; Culhane & Goldenberg, 2011; Denney & Culhane, 2009). Although the reasons for the differences in the vaginal microenvironment among various ethnicities are likely due to differences in host and environmental factors such as diet or cultural practices, these findings suggest that differences in the vaginal microenvironment may have implications in infection/inflammatory associated pregnancy outcomes, such as PTB.

Recent studies of the vaginal microbiome in pregnancy suggest that there is a reduction in taxonomic diversity of microorganisms present as pregnancy progresses (Aagaard et al., 2012). However, the factors that influence the structure and dynamics of the vaginal microbiome in pregnancy are relatively unknown. A study of 27 pregnant women found an association between oral intake of probiotics and changes in the vaginal microbiome which favored a decrease in pro-inflammatory vaginal cytokines (Vitali et al., 2012). A recent case-control study of 49 pregnant women found that the bacterial taxonomic composition of the vaginal microenvironment remained stable throughout pregnancy and that lactobacillus deficient vaginal communities were inversely associated with the gestational age of delivery. Additionally, the risk for PTB was higher in women with lactobacillus deficient communities combined with elevated levels of *Gardnerella* and *Ureaplasma* species (DiGiulio et al., 2015).

## The Complement System as a Mechanism for Preterm Birth

### Overview of the Complement System

The complement pathway is a component of the innate immune response and is so named for its primary function to "complement" the activities of antibodies in the destruction of pathogens. The pathway is composed of more than 30 soluble and membrane bound proteins which in turn initiate a series of enzymatic reactions that function to irreversibly bind to pathogens, eliminate immune complexes, and facilitate removal of products following tissue injury (Markiewski & Lambris, 2007; Sjoberg et al., 2009; Walport, 2001). Complement drives the initiation of inflammation and promotes opsonization and macrophage activation. The complement system functions at a low level steady state until its activities are amplified via activation of various pathways.

The complement activation pathway involves three pathways as described by Lynch and colleagues (Lynch et al., 2011); the classical, lectin, and alternative pathways, each of which is triggered by various initiating components. The classical pathway is triggered by the presence of bound antigen-antibody complexes; the alternative pathway is continuously activated due to the presence of foreign invaders as well as the presence of damaged self-tissues; and the lectin pathway is activated via the binding of mannose binding lectin to carbohydrate or glycoprotein groups present on the surface of microorganisms (Denny, Woodruff, Taylor, & Callaway, 2013; Lynch et al., 2011; Regal, Gilbert, & Burwick, 2015).

Regardless of the initiating component, all three pathways lead to enzymatic cleavage of complement component C3 by a pathway specific C3 convertase (enzyme), into fragments C3a and C3b. C3a is a cleavage product that functions as an anaphylatoxin, which recruits and activates inflammatory cells, amplifies local inflammation, and effects vascular permeability and smooth muscle contractility (Sjoberg et al., 2009; Walport, 2001). C3b opsonizes pathogens and/or non-self cells for destruction by phagocytic cells, and also initiates the activation of downstream complement proteins that form the membrane attack complex (C5-C9), leading to pathogen cell lysis and death (Denny et al., 2013; Lynch et al., 2011; Markiewski & Lambris, 2007). Factors B, D, and properdin lead to the activation of C3 via the alternative pathway; Bb a derivative of Factor B, is an activation product that assists in the additional cleavage of component C3 (Lynch et al., 2008). Given that complement activation products have the potential to initiate damage against self-tissues, the products are quickly removed by plasma carboxypeptides such as C3a des-Arg; factors such as

C3b and C4b are quickly deactivated and cleaved into fragments by serine proteases. Ineffective clearance may result in deposits of complement products into host tissues thereby promoting host tissue damage (Sarma & Ward, 2011).

**The Complement System in Pregnancy**

Pregnancy is a state that is associated with increased complement activation as a mechanism of both host defense and a necessary component in normal fetal/placental development (Baines, Millar, & Mills, 1974; Richani et al., 2005). In both normal and complicated pregnancies, complement products have been found in placental tissues (Faulk WP, 1980; Richani et al., 2005; Weir, 1981) and likely serve as a protective mechanism against potential infection for both the mother and fetus. Complement is involved in both fetal and placental development, as activated C3 is involved in phagocytic activities of the mouse trophoblastic invasion of the uterine vasculature (Albieri, Kipnis, & Bevilacqua, 1999). The participation of complement component C1q has also been identified in normal physiologic trophoblastic invasion of human uteroplacental tissues (Bulla et al., 2008). Components of the complement system are also important in embryonic and fetal development, as the most abundant embryotrophic factor in humans, ETF-3, contains C3, C3b, and iC3b, suggesting C3 to be important in fetal development prior to development of the placenta (Lee, Cheong, Chow, Lee, & Yeung, 2009).

Although complement has a variety of triggers for activation, dysregulation results from excess activation or poor regulation of these pathways, thereby promoting a heightened inflammatory state (Lynch et al., 2011). The uteroplacental tissues have complement regulatory proteins such as decay-accelerating factor, membrane cofactor

protein, and CD59 to prevent excessive complement activation (Holmes et al., 1990; Hsi, Hunt, & Atkinson, 1991; Liszewski, Farries, Lublin, Rooney, & Atkinson, 1996; Nishikori, Noma, Hirakawa, Amano, & Kudo, 1993). At the same time, however, deficiencies in complement components, such as C1q, C2, and C4, predispose individuals to increased risk for infection by encapsulated bacteria (Botto et al., 2009; Regal et al., 2015), as well as collagen vascular disorders (Aggarwal et al., 2010), and abnormal placentation and pregnancy complications (Singh, Ahmed, & Girardi, 2011). During early pregnancy, the classical, lectin and/or alternative complement pathways may be activated in women who have one or more additional triggers – including foreign substances or damaged tissue– as displayed in Figure 1. Excessive complement activation in response to infection or other triggers may overwhelm regulatory systems and thereby increase the risk for perinatal complications. Deposits of complement activation products have been found on various reproductive tissues including the placenta, cervix, and decidual spiral arteries (Girardi et al., 2006; Gonzalez et al., 2011).

The dysregulation of complement has been implicated in a variety of adverse pregnancy outcomes including hypertensive diseases of pregnancy (Lynch et al., 2012; Lynch et al., 2008; Lynch, Wagner, Giclas, et al., 2016), antiphospholipid antibody syndrome associated fetal loss (Breen et al., 2012), recurrent fetal loss and PTB (Lynch et al., 2012; Lynch et al., 2011; Lynch, Wagner, Deterding, et al., 2016). In the hypertensive diseases of pregnancy, antiphospholipid syndrome, and PTB studies, complement dysregulation was determined by the discovery of elevated circulating plasma levels of complement activation fragments (e.g., C3a/Bb) in the presence of the select health complication. In the studies of recurrent fetal loss and miscarriage, complement

dysregulation was determined by both elevated complement plasma levels as well as genetic deficiencies in complement regulatory genes and complement regulators (e.g., CD55), thereby resulting in increased complement activation (Mohlin et al., 2013). Complement products, such as C3a and C5a, are typically cleared quickly from the system as a result of innate regulatory mechanisms as previously described; this is essential given their effects on inflammatory responses, such as induction of chemoattraction, vasodilation, smooth muscle contraction, histamine release, and cytokine production (Sarma & Ward, 2011). As such, the identification of elevated plasma levels in select complement activation fragments in the presence of disease likely indicates some degree of dysregulation in the system.

**The Complement System in Preterm Birth: Animal Studies**

Increased levels of complement markers have shown an association with PTB in both human and animal models. In a study by Gonzalez and colleagues (2011), the administration of lipopolysaccharide (LPS) per vagina to pregnant mice resulted in cervical C3b deposits, collagen degradation, increased MMP-9 activity, and PTB (Gonzalez et al., 2011). Mice exposed to LPS that lacked a C5a receptor (C5aR-deficient) did not show cervical remodeling or increased incidence of PTB but the C5aR positive wild-type mice did. This study also found that complement activation via C5a/C5aR interactions promoted macrophage infiltration and MMP activation thereby promoting cervical ripening and PTB, which is a different mechanism of cervical ripening in term deliveries, which results from activities of cervical fibroblasts and epithelial cells (Gonzalez et al., 2011; Regal et al., 2015). Similarly, a latter study by Gonzalez (Gonzalez, Pedroni, & Girardi, 2014) found that complement activation may play a role

in myometrial contractility as elevated levels of C5a were found in the myometrium of preterm labor (PTL) mice administered vaginal LPS; these mice additionally exhibited increased expression of a contraction associated protein connexin 43.

In contrast, a similar study investigating the rates of PTB and miscarriage among C5a receptor (C5aR1) deficient knockout mice (KO) exposed to intraperitoneal LPS, found that genetic knockout of C5aR1 was associated with miscarriage but did not prevent the occurrence of PTB (Denny et al., 2015). In this study, LPS was administered into the intraperitoneal space which may more accurately mimic a systemic maternal infection as opposed to the Gonzalez study, which explored the effects of vaginally administered LPS. Although the results differ in regards to the role of the C5a receptor, they both support the theory that microbes are key factors in the etiology of PTB. The differences in study findings suggest that localized infections may work more through complement associated changes in cervical remodeling, whereas systemic exposure to LPS is likely more complex and not entirely complement dependent. (Denny et al., 2015).

**The Complement System in Preterm Birth: Human Studies**

An elevated level of C3a in the first trimester of pregnancy may be an independent predictive factor for adverse pregnancy outcomes including PTB and PPROM (Lynch et al., 2011). Specifically, women in the upper quartile of C3a were three times more likely to have an adverse outcome later in pregnancy after controlling for parity and pre-pregnancy body mass index (BMI) compared to women in any lower quartiles. In a recent biomarker discovery study, Lynch and colleagues (Lynch, Wagner, Deterding, et al., 2016) also found that the complement factors B and H along with

coagulation factors IX and IXab were the highest ranking proteins found in cases of PTB compared to term controls, suggesting that the leading pathways to PTB include the complement, immune and clotting systems. Similarly, women with elevated levels of the complement factor Bb in early pregnancy were nearly four times as likely to have PTB after controlling for known PTB related risk factors (Lynch et al., 2008). Elevated levels of amniotic fluid levels of complement factors C3a, C4a, C5a, and Bb have also been identified in women with preterm labor with microbial invasion of the amniotic cavity (Soto et al., 2009; Vaisbuch et al., 2009).

## The Microbiome and Complement Activation: A Mechanistic Model for Preterm Birth

Intrauterine infection and inflammation have been identified as definitive risk factors in the etiology of PTB, however interventions directly targeting the elimination of infection have not resulted in reducing the risk of PTB. We hypothesize that the risk may not be reduced because there may be additional pathogenic bacterial species present that stimulate inflammatory pathways, some of which which may not be detectable with traditional culture based methods, sensitive to standard therapies, or associated with overt clinical infection. Thus the analysis of samples collected from various sites using 16S DNA sequencing could allow for the identification of a greater number of potentially virulent microbial species. Evaluation of the composition and metabolic potential of the human microbiome along with complement markers may provide a more comprehensive evaluation of the community of microorganisms present in various locations of the human body and may elucidate their relationship to the onset of inflammation, and the increased risk for preterm birth.

In Figures 1, we present a model of a hypothesized microbiome-complement

activation pathway to PTB. We posit that pathogenic microbes from various locations (e.g., vaginal, oral, respiratory, gut, placental) influence the activation of complement pathways (e.g., C3a and Bb) in the maternal vasculature and reproductive tissues. Although complement proteins are found throughout the body without adverse effects, during infection the level of complement activation is increased to assist in the osponization (tagging) and elimination of pathogens via phagocytes (e.g., macrophages). We hypothesize that the composition of the microbiome in the vagina, but elsewhere as well, will influence the degree to which the complement system is activated. The resulting inflammatory response involves the production of various inflammatory mediators, which if dysregulated may break down collagen, stimulate uterine contractions, facilitate cervical ripening, and promote decidual activation, thereby increasing the risk for PTB.

Although regulatory mechanisms are present to prevent uncontrolled complement activation, the system may become overwhelmed pending the number of factors present. Complement pathway dysregulation (defined as excess activation or poor regulation) promotes not only destruction of pathogens, but may also cause injury to self tissues (Girardi et al., 2006), as deposits of complement activation products have also been found on the placenta, cervix, and decidual spiral arteries (Gonzalez et al., 2011). If complement attaches to these reproductive tissues, then macrophages can likewise attach via their complement receptors and begin to degrade the tissue, which may be another pathway to PTB (Figure 1). We propose that women who have one or more triggers for complement activation including foreign substances, damaged tissue, and/or infection may be prone to complement dysregulation, thereby overwhelming regulatory systems

and increasing the risk for perinatal complications via direct injury from the effects of complement dysregulation in the reproductive tissues or via effects of the resulting inflammatory response, as previously described.

**Timing of Complement Dysregulation and Preterm Birth**

We propose that the dysregulation of maternal complement activation at any point across gestation (implantation-37 weeks gestation) may increase the risk of PTB, as shown in Figure 2. During early pregnancy there is normal physiologic activation of complement to prevent infection at the maternal-fetal interface and to promote the clearance of tissue debris resulting from implantation and placental development; this last task, clearing of tissue debris, is essential for successful trophoblast invasion. Coupled with early pregnancy infection and/or shifts in the microbiome composition, however, there may be excess activation of complement. Dysregulation of complement during placental development or early pregnancy may promote an altered inflammatory profile more favorable for the development of inflammatory  adverse pregancy outcomes such as PTB. This hypothesis is supported by studies that have shown that elevated levels of 1$^{st}$ trimester complement factor C3a and Bb have been associated with PTB and PPROM (Lynch et al., 2008; Lynch et al., 2011). Similarly, we propose that dysregulation of maternal complement could also occur later during gestation as a result of similar factors including infection, shifts in the microbiome composition, or normal physiologic activation.

## Conclusions

The relationship between infection, preterm birth, and preterm premature rupture of membranes suggests a major role for both the microbiome and complement

dysregulation in the biological pathway to PTB. The presence of pathogenic microbes in low abundance in the absence of clinical symptoms may promote the activation of complement and increase the risk for PTB (Ravel et al., 2011). We hypothesize, therefore, that the infection associated pathway to preterm birth is not simply due to the presence and activities of pathogenic microbiota, but rather is more complex and may involve the combination of pathogenic microbes present, the ratio of pathogens to one another, the number of "protective" species present in the environment, and the individual woman's inflammatory – and complement -- response to these factors.

In summary, intrauterine infection and the activation of pro-inflammatory processes in the intrauterine environment are definitive risk factors for preterm birth. Although intrauterine infection and/or inflammation are strongly associated with PTB, the immunological response to infection may be influenced by immunogenetic factors, such as gene polymorphisms or gene-environment interactions (Moura et al., 2009), which may make some exposed women more susceptible to inflammation-associated PTB. Failure to identify the most susceptible women may help to explain why the treatment of infection has not resulted in decreased PTB risk. Also, some women may be infected with previously unidentifiable microbes. Newer culture-independent PCR methods have allowed for the identification of an even greater number of microbes not typically found with culture dependent methods via identification of the 16S ribosomal rRNA gene, thereby allowing for a more detailed DNA based evaluation of the microbiota present (Aagaard et al., 2012; Eckburg et al., 2005; Lane et al., 1985). Using these newer methods to explore routes associated with intrauterine infection, such as the vaginal, oral, gut, or even skin microbiome, in conjunction with measures of complement

activation, may elucidate a potentially modifiable biobehavioral pathway of inflammation-associated preterm birth.

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# Figures

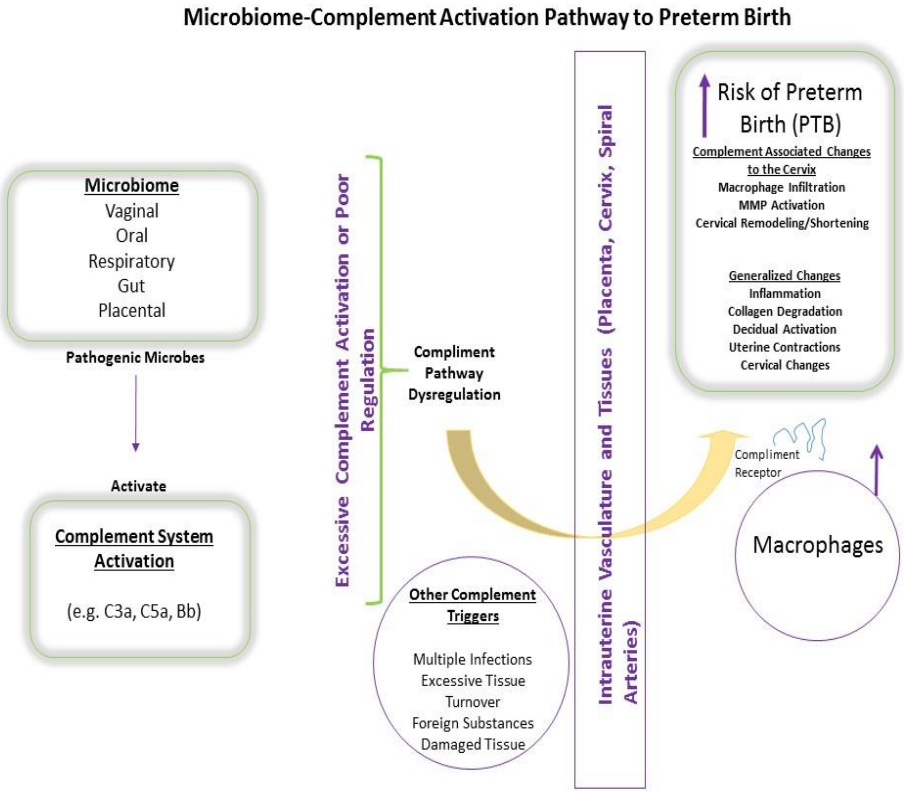



**Figure 2.1**- Microbiome and complement dysregulation pathway to preterm birth. The composition of the microbiome may influence the activation of complement pathways in the maternal vasculature and reproductive tissues. We hypothesize that complement dysregulation in the intrauterine environment enhances inflammation and the production of inflammatory mediators, which in turn promote direct changes to the cervix, collagen degradation, activation of the uterine decidua, and uterine contractility, thereby increasing the risk for preterm birth.

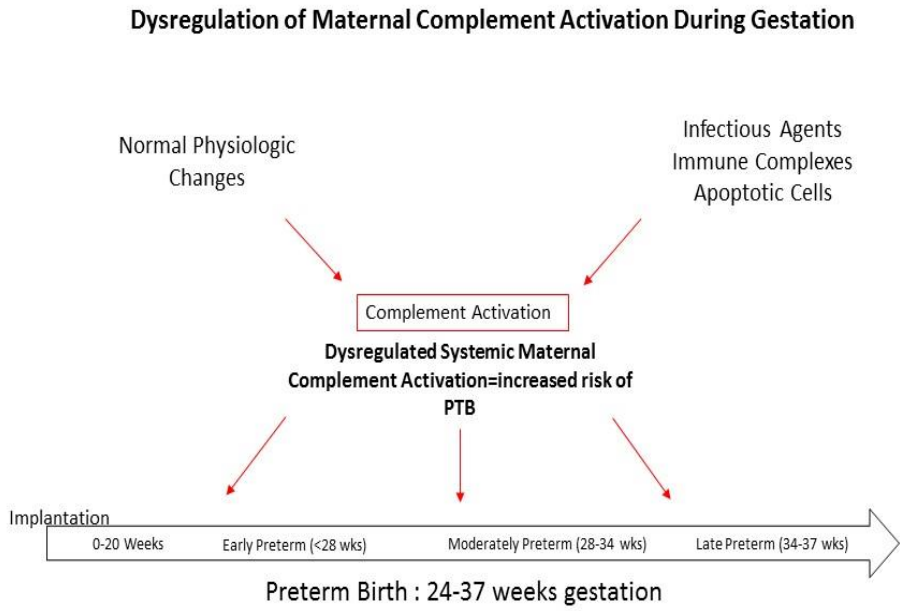



**Figure 2.2- Dysregulation of Maternal Complement Activation During Gestation.** The dysregulation of maternal complement activation at any point across gestation (implantation-37 weeks gestation) may increase the risk of PTB.

**Figure 2.2- Dysregulation of Maternal Complement Activation During Gestation.** The dysregulation of maternal complement activation at any point across gestation (implantation-37 weeks gestation) may increase the risk of PTB.

# Chapter 3:
# Complement System Activation and the Gestational Vaginal Microbiome in African American Women

## ABSTRACT

**Introduction**: The purpose of this study was to explore the relationship among the composition of the vaginal microbiome, reproductive tract infection, and biomarkers of complement activation (C3a and Bb) during early pregnancy. Recent findings suggest that complement-associated inflammatory events during early pregnancy are associated with an increased risk for preterm birth (PTB). Studies have shown that African American (AA) women experience a disproportionate rate of inflammation associated PTB as well as elevated rates of vaginal infection with microbes highly associated with PTB. This study sought to explore measures of complement system activation in relation to the vaginal microbiome, a microbial environment shown to harbor bacterial populations strongly associated with an increased risk for PTB.

**Methods:** A first trimester plasma sample was collected from 144 pregnant African American women enrolled in a larger prospective study exploring the biobehavioral risk factors for PTB and the microbiome in African American women. The sample was collected between 8 and 14 weeks gestational age for later analysis of C3a and Bb concentrations and was stored at -80C along with a self-collected vaginal swab obtained at the exact same time point to evaluate vaginal microbiome composition. Chart review was conducted to collect information regarding recent history of reproductive tract infection.

**Results:** The mean plasma concentration for C3a in early pregnancy was (4001.42±118.77 µg/ml) and the mean concentration for Bb was (1.48±0.05 ng/l). The vaginal microbiome composition of women in the sample clustered into five distinct community state types, each dominated by one of the following microbes: (1) CST 1 *Lactobacillus*; (2) CST 2 *Prevotella/Bacteroides*; (3) CST 3 Snethia/Gardnerella; (4) CST 4 *Lactobacillus iners*; (5) CST 5 *Shuttleworthia*. Linear regression analyses concluded that the vaginal microbiome composition, characterized by community state type, was not associated with early pregnancy C3a or Bb concentrations; similarly, reproductive tract infection was not associated with C3a or Bb concentrations.

**Discussion:** This study suggest that the microbes of vaginal microbiome classified by CST as well as reproductive tract infection are not factors associated with elevated C3a and Bb concentrations during early pregnancy. The identification of population specific inflammatory mechanisms associated with PTB in AA women may prove beneficial in the development of prevention and management strategies to eliminate the health disparity in PTB rates among AA women, however more studies are needed to identify factors that are associated with early pregnancy complement system dysregulation and the relationship with poor pregnancy outcomes.

Keywords: Vaginal Microbiome; complement system; pregnancy; preterm birth

## INTRODUCTION

Preterm birth (PTB), defined as birth prior to 37 weeks gestational age, is a complication of pregnancy that is multifactorial and syndromic in nature; however, there is strong evidence that suggests that dysregulation in the activities of the immune system may be a key risk factor contributing to the underlying pathology of the syndrome (Bastek, Gomez, & Elovitz, 2011; Romero, Gotsch, Pineles, & Kusanovic, 2007). Racial disparities persist among African American (AA) women who are 1.5 times as likely to have PTB (16.8%) as Caucasian (10.8%) or Asian (10.4%) women (Dimes, 2014; Hamilton, Hoyert, Martin, Strobino, & Guyer, 2013). There are also racial disparities in the occurrence of inflammation-associated PTB, with AA women more likely to be affected (Hitti J, 2007). Innate and adaptive immune mechanisms evolve during pregnancy to promote survival of the semi-allogenic fetus while simultaneously protecting the mother from attack by foreign invaders (Mor & Cardenas, 2010), however exaggerated production and/or poor regulation of inflammatory mediators can have deleterious effects on various blood vessels and tissues, resulting in a host of pregnancy complications including PTB (DiGiulio, 2012; Romero et al., 2006).

One such mechanism, the complement system, functions at the implantation site to ensure successful trophoblast invasion and embryo implantation, which are critical processes in normal placental and fetal development (Pfeifer, Kawahara, & Hugli, 1999). The complement system also enhances the activities of antibodies and phagocytic cells (e.g. macrophages) in the process of eliminating bacteria and damaged cells from the body (Frank, 2000; Sarma & Ward, 2011). The process of complement activation is highly complex and involves biochemical activation via three pathways: the classical,

alternative, and lectin pathways, which are described elsewhere (Lynch et al., 2011; Sjoberg, Trouw, & Blom, 2009). Upon activation, complement proteins are cleaved by select protease enzymes in a cascade-like fashion, resulting in inflammation, attraction of phagocytic cells, and formation of the membrane attack complex (MAC), thereby eliminating foreign and/or damaged cells (Tegla et al., 2011).

Dysregulation in the activities of the complement system have been associated with a variety of adverse pregnancy outcomes. Recent studies have shown that elevated concentrations of complement fragments Bb and C3a early in pregnancy are associated with increased risk for the development of hypertensive disorders of pregnancy as well as spontaneous PTB (Lynch et al., 2012; Lynch et al., 2008; Lynch et al., 2011; Lynch, Wagner, Deterding, et al., 2016; Lynch, Wagner, Giclas, et al., 2016) in predominantly Caucasian populations. The activities of the complement system are tightly controlled by a variety of soluble and membrane-associated inhibitor mechanisms that control the level of activation (Pfeifer et al., 1999). Dysregulation in the activities of the complement system can promote an exaggerated inflammatory response and/or host tissue damage; however, the factors that contribute to early pregnancy elevations in complement levels and subsequent adverse pregnancy outcomes are not fully understood.

The purpose of this study is to explore the relationship between the complement system and bacterial populations of the vaginal microbiome, a microbial environment shown to harbor bacterial populations strongly associated with an increased risk for PTB. Specifically, we seek to explore the relationship between C3a and Bb with the vaginal microbiome classified by community state type as well as the relationship with clinically diagnosed reproductive tract infection (e.g. bacterial vaginosis, chlamydia, gonorrhea,

urinary tract infection, and trichomoniasis) in the population most at risk for adverse pregnancy outcomes; African American women. The identification of a complement-microbiome biological pathway may help to better understand the mechanisms by which microbes promote dysfunction in the reproductive tissues, thereby increasing the risk for inflammatory-driven adverse pregnancy outcomes such as PTB, particularly among AA women.

## BACKGROUND

### Microbial Triggers of Complement Activation

Intrauterine infection and inflammation are thought to be stimulated by the presence of pathogenic microbes in the intrauterine and cervical environment (DiGiulio, 2012; Marconi, de Andrade Ramos, Peracoli, Donders, & da Silva, 2011), which are thought to ascend from the vaginal microenvironment. However, there is also evidence for hematogenous spread of microbes from other sites (Aagaard et al., 2014; Subramaniam et al., 2016). Although microbes from any body site could potentially stimulate complement activation pathways, ascension of bacteria from the vaginal microenvironment into the reproductive tissues is cited as the most common pathway and immediate route for intrauterine infection and inflammation (Romero, Dey, & Fisher, 2014), key risk factors for PTB. The complement system is heavily involved in the host immune response against microbial pathogens, suggesting that dysregulation in the activities of the complement system may promote an ineffective immune response against infection.

Foreign invaders, such as bacteria, have long been implicated in the development of PTB, likely via the activation of inflammatory mediators (e.g. cytokines,

prostaglandins, matrix metalloproteinases) in the reproductive tissues; these processes stimulate the myometrium, weaken the amniotic membranes, and alter the strength of the cervix (Holst, Mattsby-Baltzer, Wennerholm, Hagberg, & Jacobsson, 2005; Romero et al., 2007). PTB is a multifactorial syndrome of pregnancy with many potential etiologic mechanisms, but intrauterine infection and inflammation resulting from microbial invasion of the amniotic cavity is associated with nearly 25-50% of PTB cases (Tambor et al., 2015). Several studies investigating the relationship between microbial invasion of the amniotic cavity (MIAC) and risk for PTB have shown that greater microbial invasion is associated with a slightly higher risk for PTB and preterm premature rupture of membranes (Cobo et al., 2013; DiGiulio, 2012; Goldenberg, Hauth, & Andrews, 2000; Marconi et al., 2011), suggesting that the presence of microbes in the intrauterine environment may trigger the activation of inflammatory processes that also play a role in the activation of labor physiologic pathways.

**The Vaginal Microbiome**

Exposure to bacteria during pregnancy is inevitable as microbes are a normal component of the human ecosystem (Turnbaugh et al., 2007). The human "microbiome" refers to the total sum of commensal and pathogenic microorganisms that live on and in the human body (Peterson et al., 2009). The microbial community of the vagina is diverse and consists of more than 50 species of bacteria that vary between women depending on host and environmental factors (Cribby, Taylor, & Reid, 2008; Ravel et al., 2011). The vaginal microenvironment is maintained in a healthy state by the presence of protective bacteria such as *Lactobacillus*, which helps to limit growth of pathogenic organisms by producing bacteriostatic compounds and promoting an acidic environment via lactic acid

production (Kaewsrichan, Peeyananjarassri, & Kongprasertkit, 2006; Linhares, Summers, Larsen, Giraldo, & Witkin, 2011). The vaginal microbiome has been described primarily using six community state types (CSTs). Four CSTs are characterized by *Lactobacillus* dominance, while the other two are dominated by anaerobic species commonly associated with infection (Ravel et al., 2011; Zhou et al., 2007). The factors contributing to shifts in the composition of the vaginal microbiome are largely unknown, but differences related to hormone balance and gene-environment interactions have been proposed.

Early studies of the vaginal microbiome signature during pregnancy suggest that the ecosystem during pregnancy is relatively stable, although as pregnancy progresses the diversity of the vaginal microbiome decreases (Aagaard et al., 2012). A recent retrospective case-control study of predominantly Caucasian women found that pregnant women were more likely to have CSTs dominated by protective bacterial species *Lactobacillus vaginalis, L. crispatus, L. gasseri,* and *L. jensii,* and less likely to have CSTs associated with high relative abundance of *Prevotella, Sneathea, Gardnerella, Ruminococcaceae, Parvimonas,* and *Mobiluncus,* which are taxa more commonly associated with bacterial vaginosis (Romero, Hassan, et al., 2014). A recent study of the gestational vaginal microbiome found that African American women who delivered preterm had less bacterial diversity in their vaginal microbiome with a lower relative abundance of Sneathia and Prevotella compared to AA term controls (Nelson, Shin, Wu, & Dominguez-Bello, 2016). Bacterial vaginosis, which results from a shift in the vaginal microenvironment that favors the growth of anaerobes, is associated with increased risk of intrauterine infection and PTB (CDC, 2016; Denney & Culhane, 2009; Leitich & Kiss, 2007).

Despite the risks associated with colonization of the vagina by select microbes, elimination of infection has not been found to decrease the incidence of PTB (Bastek et al., 2011). Additionally, it is not fully understood why some women develop ascending intrauterine infection and subsequent PTB in the presence of select microbes whereas other women have no complications. These differences imply that exploring differences in host inflammatory responses to microbes may help to better understand the underlying biologic pathways of infection-associated PTB among high risk populations.

Given the role of the complement system in host immunity, key pregnancy physiologic processes, and the development of adverse pregnancy outcomes, the aim of this study was to explore the relationship between the composition of the vaginal microbiome classified by CST and measures of complement activation (C3a and Bb) among AA women during early pregnancy. Two hypotheses were tested: (1) women with CSTs dominated by primarily pathogenic bacteria would demonstrate higher plasma concentrations of C3a/Bb compared to women with CSTs dominated by healthier bacteria; and (2) women with reproductive tract infection during early pregnancy would demonstrate higher plasma concentrations of C3a/Bb. Despite the association of select microbes with an increased risk for select adverse pregnancy outcomes, the underlying pathologic mechanisms are not well understood. The identification of biologic risk factors associated with excessive complement system activation during early pregnancy may prove beneficial in the development of more effective preventive and early diagnostic strategies targeting PTB in AA women.

# METHODS

## Sample and Setting

A socioeconomically diverse cohort of 144 pregnant AA women were followed prospectively in this study to explore the relationship between the vaginal microbiome CSTs and reproductive tract infection with early pregnancy complement activation biomarkers. African American (AA) women enrolled in a larger study investigating the "Microbiome and Biobehavioral Risk Factors for Preterm Birth in AA women" were selected for inclusion in this analysis. The dissertation study was approved under the parent study general IRB-approved investigations of "inflammatory markers" included in the informed consent, such that a separate consent for the measurement of complement markers was not needed. The sample selected for the dissertation study was selected from a larger group of 184 women actively enrolled in the parent study who had an estimated date of delivery prior to the end of March 2016, and for whom 16s microbiome sequencing data would be available. Data and samples collected from a subset of 144 women who also had plasma available for analysis of complement levels were used to achieve the Aims identified in this dissertation study.

Pregnant AA women were recruited during their 1$^{st}$ trimester of pregnancy (2014-2016) via outreach to local prenatal clinics associated with a public and a private hospital in Atlanta Georgia. After an initial screening, those who met inclusion criteria and indicated willingness to participate in the larger study, were asked to provide informed consent and then enrolled. The recruitment and enrollment processes for the parent study as well as inclusion and exclusion criteria are described in greater detail

elsewhere. (Corwin et al, 2017). The study was approved by the Emory Institutional Review Board.

Women completed a series of demographic and behavioral questionnaires including questions related to vaginal hygiene practices, sexual practices, and other health behaviors experienced in the last month. Stored plasma samples as well as vaginal microbiome samples collected as a part of the larger parent study protocol were used for analysis. A detailed review of the medical record post-delivery was conducted to collect sociodemographic data and information on clinical outcomes of interest (e.g. infection history).

## Specimen Collection and Processing

### *Complement Biomarkers C3a and Bb*

Plasma concentrations of the complement fragments C3a and Bb were measured from samples collected during the first visit of the larger Study. Each sample on arrival to the lab was centrifuged and aliquoted within an average time of 30 minutes and stored in a freezer at -80C. At the time of analysis, plasma supernatant samples were thawed and batch analyzed for measurement of complement C3a and Bb concentrations. C3a was measured using an enzyme-linked immunosorbent assay (ELISA) kit (BD Opt EIA, BD Biosciences, CA). Plasma concentrations of the Bb fragment were measured with MicroVue Bb Plus Fragment EIA kit (Quidel Corporation, OH). Plasma samples were loaded onto pre-coated antibody microtiter plates that were specific to the complement fragment of interest and processed in duplicate according to the manufacturer's recommendations. The absorbance readings were measured using a Synergy 4 Microplate

Reader (BioTek, VT) at 450 nm. Gen5 curve-fitting statistical software (BioTek, VT) was employed for calculation of ELISA results. The inter-assay (plate to plate) coefficients of variation (CV%) were 18.1 for C3a and 5.3 for Bb; the intraassay (within plate) coefficients of variation were 5.8 for C3a and 17.6 for Bb.

*Vaginal Microbiome*

Women provided self-collected vaginal microbiome samples as described in the collection procedures of the parent study protocol (Corwin et al, 2017). DNA was extracted using the MoBio PowerSoil Isolation Kit. The V3-V4 region of the 16S rRNA gene was PCR-amplified and sequenced on an Illumina MiSeq platform. Paired-end reads were joined with PANDASeq using default options, resulting in a median of 117,109 reads per sample (range: 19,304 to 379,100). We used Quantitative Insights Into Microbial Ecology (QIIME) version 1.9.0 to perform closed-reference OTU-picking against the greengenes database version 13_8. To calculate measures of microbiome diversity (e.g. Shannon and Chao1 alpha diversity metrics), we rarefied all samples to the number of sequences in the sample with the fewest number of reads and calculated the metrics in R using the vegan package. For each sample, we used the median of 10,000 such rarefactions in analysis. Weighted UniFrac distances (a measure of between-sample diversity) were calculated, and this information was used to generate a phylogenetic tree of samples QIIME, which we visualized using FigTree v1.4.2. The relative abundance of each OTU was utilized to classify samples from each woman into community state types using methods previously described by Digiulio (DiGiulio et al., 2015). Briefly, Bray-Curtis distance metrics were calculated and used for multidimensional scaling and

denoising. Samples were clustered into five community state types, consistent with previous studies. (Ravel et al., 2011).

**Statistical Analysis**

Descriptive statistics (e.g. mean, median, standard deviation and percentages) were calculated for demographic (e.g. age, BMI, insurance status, race relationship status), biological (complement levels and vaginal microbiome measures), and clinical variables of interest (infection history). Relationships between variables were explored using Pearson correlation, Student t-tests, Kruskal-Wallis, ANOVA, Chi-square, and Fisher's exact tests. Kolmogorov-Smirinov test was used as a test for normality of continuous variables. Controlling for select covariates, multivariable linear regression analysis was used to explore the relationship between the vaginal microbiome community state types and reproductive tract infection with complement concentrations (the dependent variable in all analyses). The data were analyzed in SPSS 24.0. Statistical tests were two-sided with a p-value<0.05 used to determine statistical significance.

## RESULTS

**Descriptive Statistics**

A total of 144 women were included in the study. Table 1 outlines the baseline sample characteristics for the sample. The median age of women in the study was 23.5±6.0 years with a median early pregnancy BMI of 26.1±10.9. The majority of the sample was Medicaid insured (70.8%), although 54% were educated at the college level or higher. Most women in the sample were single (86.8%); however, 50.7% reported being in a relationship in which they cohabitated with a partner.

Table 2 outlines the complement marker concentrations, vaginal microbiome metrics, and clinical outcomes for women in the sample. The mean concentration of complement marker C3a was 4001.43±118.77 ng/mL and 1.48±0.05 μg/mL for Bb; the distributions of the biomarkers were relatively normal are within reasonable limits of C3a and Bb levels reported in the literature (Lynch et al., 2011; Lynch, Wagner, Deterding, et al., 2016; Richani et al., 2005). The vaginal microbiome metrics including the shannon diversity index (SDI), chao 1, and community state types (CST) are also described in table 2. The mean SDI was 2.10±1.2 and the mean Chao 1 was 196.68 ±177.49, which are both consistent with literature findings (Hyman et al., 2014; Nelson et al., 2016). The microbial communities clustered together into five distinct community state types as shown in the heatmap (figure 1): (1) CST 1 had a high relative abundance of the traditionally healthy type *Lactobacillus*; (2) CST 2 had the lowest levels of *Lactobacillus* with a higher relative abundance of *Prevotella*, *Bacteroides*, and *Porphyromonas*; (3) CST 3 had a high relative abundance of *Sneathea* and *Gardnerella*; (4) CST 4 was dominated by *Lactobacillus iners*; (5) CST was dominated by *Shuttleworthia*. There was a relatively even distribution of the women across the five CST's (Table 2). The most common clinically diagnosed reproductive tract infections were Bacterial vaginosis (BV), urinary tract infection (UTI), and chlamydia (Table 2). BV is a type of vaginal condition characterized by overgrowth of pathogenic microbes such as *Gardnerella, Bacteroides, and Prevotella* (Oakley, Fiedler, Marrazzo, & Fredricks, 2008); in the cohort there were no women with BV in CST 1, while the highest percentage of women with BV were in CST 2 and CST 3.

Figure 2 provides a second heat map of the microbiome composition classified by CST along with the associated complement concentration; women who were above the median concentration of C3a and Bb are designated in yellow and women below the median concentration are designated blue. There are several subjects with an elevated concentration of complement above the median that have corresponding microbiome CST profiles dominated by infection associated microbes including those found in CST 2 and 5.

**Univariate Associations**

Univariate associations between the key independent variables (CST, reproductive tract infection) and other covariates (e.g. age, education level) are outlined in the top portion of table 3. There were no significant relationships identified between select variables and CST. There were statistically significant associations between age and reproductive tract infection <20 weeks ($t=3.05$; $p=0.003$). Women diagnosed with a reproductive tract infection less than 20 weeks were younger. There was also a statistically significant association between infection < 20 weeks and insurance status ($\chi^2=12.93$; $p=0.000$). Of the 49 women with vaginal infection less than 20 weeks, 44 (89.8%) of them were insured by Medicaid compared to 5 (10.2%) who were privately insured. There is also a statistically significant association between infection < 20 weeks and marijuana use ($\chi^2=5.19$; $p=0.02$). Of the 21 women who smoked marijuana, 12 (57.1%) of them had a vaginal infection <20 weeks compared to 9 (42.9%) of them who did not.

Associations between the independent variables (CST, reproductive tract infection) and co-variates (e.g. age, education level) with the dependent variables (C3a

and Bb) are outlined in bottom half of Table 3. There is a statistically significant association between C3a concentration and age (r=0.34; p=0.000), gestational age at sample acquisition (r=0.20; p=0.02), maternal education level (t=-3.04; p=0.003), and insurance type (t=-2.39; p=0.02). As a woman's age and gestational age at the time of sample collection increases, the concentration of C3a levels also increase. Women who have received some college education or greater had a higher mean concentration of C3a (4142.56± 126.96 ng/mL) compared to women with a high school education or less (mean=3126.39± 265.89ng/mL). Similarly, women with private insurance had a higher mean concentration of C3a (4436.02± 218.99ng/mL) compared to women who were Medicaid insured (3822.47±138.18 ng/mL). There was also a significant relationship identified between the type of health insurance and Bb levels (p=0.02); women insured by Medicaid had a higher mean concentration of Bb (1.54±0.06 mg/mL) compared to women who were privately insured (1.31±0.07 ng/mL).

**Regression Analyses**

*CST*

Table 4 presents the regression models for CST with complement markers C3a and Bb. Controlling for select covariates, there was a significant relationship identified between CST and C3a ($F=9.69$, $p<0.001$, $R^2=0.52$). The only significant variable identified in the model was the assay plate variable, which was included to control for the high interassay (plate to plate) variability that occurred during C3a assay analysis. To reduce the influence of the assay plate variable, C3a analyses were repeated using only women with less than 10% variation in concentration between the sample duplicates. There remained a significant relationship identified between C3a and the other covariates

taken together (F=2.68, p=0.006, $R^2$=0.19), however CST was not a significant predictor (p=0.65). The only significant covariate identified was age (p=0.02) For every one year increase in age, the C3a concentration would be expected to increase by 75.83 units.

Linear regression analysis indicated no significant relationships between CST and Bb (F=1.60, p=0.13, $R^2$=0.09). Interassay variability was in an acceptable range for Bb (<10%), however similar quality control measures were also applied to the Bb regression models; women with >10% variation in sample duplicates were removed to remain consistent in analysis. There were no additional relationships identified between CST and Bb and the covariates taken together. (F=1.14, p=0.35, $R^2$=0.11).

*Reproductive Tract Infection*

Table 5 presents the regression models for reproductive tract infection with complement markers C3a and Bb. There was a significant linear relationship identified between reproductive tract infection and C3a (F=3.72, p=0.001, $R^2$ =0.17), however reproductive tract infection was not a significant variable (p=0.51). Age (p=0.006) was the only significant covariate in the model (Table 5). There were no significant linear relationships identified between reproductive tract infection and Bb (F=1.52, p=0.18, $R^2$=0.11).

## DISCUSSION

The purpose of this study was to explore the relationships between composition of the vaginal microbiome, reproductive tract infection, and biomarkers of complement activation (C3a and Bb) among a population of African American women during early pregnancy. Our findings do not indicate any significant relationships between vaginal microbiome community state type, reproductive tract infection, and markers of

complement activation. If elevated concentrations of the complement biomarkers C3a and Bb are associated with a greater risk for adverse pregnancy outcomes as previously reported in the literature for predominantly white populations (Lynch et al., 2008) (Lynch et al., 2011), other mechanisms that promote elevated complement activation are likely as our findings demonstrate that the vaginal microbiome and reproductive tract infection do not have a significant influence on complement levels.

Age appears to be an important factor as it relates to complement C3a concentrations. Findings in our study show that as age increases complement C3a concentrations increase. C3a is a potent inflammatory mediator and marker commonly associated with the classical complement pathway, which involves the more traditional immune response associated with antigen/antibody binding. It could be that women who are older demonstrate a more robust classical immune response as evidenced by the higher levels of the C3a marker for every one year increase in age. This finding may also indicate that there are age related changes and potential weathering in the activities of the complement system that promote a heightened inflammatory state. Age associated changes in immune and inflammatory responses have been previously reported in African American women (Geronimus et al., 2010), suggesting that aging may be associated with dysregulation in immune and inflammatory responses resulting in increased inflammation (Woods, Wilund, Martin, & Kistler, 2012). In pregnancy, it has been shown that women with a very young or advanced maternal age may have an increased risk for adverse pregnancy outcomes (Jacobsson, Ladfors, & Milsom, 2004; Kenny et al., 2013), however more studies would be needed to confirm if C3a concentrations and age are associated with adverse pregnancy outcomes.

Others have explored the role of the complement system as it relates to adverse pregnancy outcomes and have shown that elevated levels of C3a and Bb are associated with poor outcomes (Lynch et al., 2008; Lynch et al., 2011; Lynch, Wagner, Deterding, et al., 2016). Although these findings are significant each of the studies were composed of white women with rates of enrollment for AA women ranging from 5-7%. Racial differences in the function of the complement system may be plausible as previous studies have demonstrated that there are racial differences in immune and inflammatory function, particularly among African American women (Corwin et al., 2013).

**Limitations**

The complement system is a highly complex system with several triggering factors including pathogen exposure as well as activation via exposure to products associated with tissue injury including mitochondrial membranes, immune complexes, and apoptotic bodies (Lynch et al., 2008). The soluble factors of the complement system are not only located in the circulation, but also reside in host tissues ready to activate for both endogenous (e.g. tissue injury/cell breakdown) as well as exogenous triggers (e.g. infection) (Markiewski & Lambris, 2007). Dysregulation typically occurs as the result of genetic factors, excess activation due to the presence of multiple stimulating factors, or ineffective control by host regulatory mechanisms. (Lynch et al., 2008; Pfeifer et al., 1999). As such, exploring only the vaginal microbiome as it relates to complement system activities may not be effective in understanding how the system becomes dysregulated. Future studies may benefit from an approach that explores multiple risk factors for complement activation with the goal of creating risk profiles that take into account a variety complement triggers, while simultaneously exploring the association with adverse pregnancy outcomes.

The study also had challenges related to the measurement of the complement markers. Specifically, the interassay variability was elevated for C3a above the recommended 15% or less. Variations in the measurement of biomarkers is often attributed to several factors including the experience of the person conducting the assays, poor adherence to the assay protocol, or issues related to storage of the samples. Although complement can be measured from stored samples, the assay procedures have to be followed very carefully as spontaneous activation of complement can occur if samples are not handled correctly or accurately processed according to the assay protocol (Pfeifer et al., 1999).

## CONCLUSIONS

The complement system is a key component of innate immunity which assists in the process of destruction and clearance of select targets (Frank, 2000; Sjoberg et al., 2009), but is also a necessary component of early pregnancy activities including implantation, vascular remodeling, and placental development (Bulla, Bossi, & Tedesco, 2012; Lynch et al., 2012; Lynch et al., 2011; Sjoberg et al., 2009). Although there are a variety of triggers for complement system activation, dysregulation is associated with an exaggerated inflammatory state as a result of effects of the activities of complement proteins (e.g. C3a, Bb). Although early pregnancy complement associated immune and inflammatory events have been associated with an increased risk for adverse pregnancy outcomes including PTB, little is known about intra-pregnancy risk factors for complement activation. This study demonstrates that the microbes of vaginal microbiome classified by CST as well as reproductive tract infection are not factors associated with elevated concentrations complement activation fragments C3a or Bb. The identification

of population specific inflammatory mechanisms associated with PTB in AA women may prove beneficial in the development of prevention and management strategies to eliminate the health disparity in PTB rates among AA women, however more studies are needed to identify factors that are associated with early pregnancy complement system dysregulation and the relationship with poor pregnancy outcomes.

## Tables and Figures

**Table 1: Sample Characteristics (N=144)**

| Sociodemographics | Mean (SD), Median (IQR), or n (%) |
|---|---|
| **Age, median (IQR)** | 23.5 (6.0) |
| **Insurance Status, n (%)** | |
| Medicaid | 102 (70.8) |
| Private | 42 (29.2) |
| **Race, n (%)** | 144 (100) |
| African American | |
| **Education Level, n (%)** | |
| High School or less | 66 (45.8) |
| College or greater | 78 (54.2) |
| **Marital Status, n (%)** | |
| Married | 19 (13.2) |
| Single | 125 (86.8) |
| **Relationship Status, n (%)** | |
| not in a relationship | 28 (19.4) |
| in a relationship (no cohabitation) | 43 (29.9) |
| in a relationship (cohabitation) | 73 (50.7) |

Data are presented as mean ±standard deviation or standard error, median (IQR), or n (%)

**Table 2: Complement/Microbiome Measures and Clinical Outcomes (N=144)**

| Complement Measures | Total Cohort[B] |
|---|---|
| **C3a (ng/mL), mean ±SE (median)** | 4001.43± 118.77 (3781.51) |
| **Bb (µg/mL), mean ±SE (median)** | 1.48± 0.05 (1.37) |
| **Microbiome Measures** | |
| **Community State Types (CST), n (%)[A]** | |
| **I** | 21(14.7) |
| **II** | 34 (23.8) |
| **III** | 28 (19.6) |
| **IV** | 38 (26.4) |
| **V** | 22 (15.4) |
| **Shannon Diversity Index (SDI), mean± SD** | 2.10 ±1.16 |
| **Chao 1, mean± SD (median)** | 196.68 ±177.49 (121.0) |
| **Clinical Outcomes** | |
| **BMI at 1st prenatal visit, median (IQR)** | 26.1 (10.9) |
| **Gestational age at sample collection, median (IQR)** | 11.6 (3.3) |
| **Infection prior to 20 weeks, n (%)** | |
| Bacterial Vaginosis | 22 (15.3) |
| Urinary Tract Infection | 15 (10.4) |
| Chlamydia | 12 (8.3) |
| Trichomoniasis | 7 (4.9) |
| Gonorrhea | 1 (0.7) |
| Candidiasis | 1 (0.7) |

[A] One participant declined to provide a vaginal microbiome sample, N=143

[B] Data are presented as mean ±standard deviation or standard error, median (IQR), or n (%)

**Table 3: Associations Between Independent Variables, Covariates, and Dependent Variables**

| Covariates | Independent Variables | |
|---|---|---|
| | **CST** | **Infection <20 weeks** |
| Age | p=0.16[A] | t=3.05; p=0.003* |
| GA at sample acquisition | p=0.49[A] | t=-0.08; p=0.94 |
| Maternal education | $\chi^2$=7.50, p=0.11[B] | $\chi^2$=2.64; p=0.10 |
| Insurance status | $\chi^2$=5.96; p=0.20[B] | $\chi^2$=12.93; p=0.000* |
| BMI | p=0.77A | t=-0.33; p=0.74 |
| Cigarette smoking | $\chi^2$=1.07; p=0.89[B] | $\chi^2$=1.54; p=0.17 |
| Marijuana Use | $\chi^2$=5.25; p=0.26[B] | $\chi^2$=5.19; p=0.02* |
| Parity | $\chi^2$=5.48; p=0.24 | $\chi^2$=0.17; p=0.68 |
| | | |
| | **Dependent Variables** | |
| **Independent Variables** | **C3a continuous** | **Bb continuous** |
| CST | F=0.49; p=0.74 | F=0.20; p=0.94 |
| Reproductive tract Infection <20 weeks | t=0.68; p=0.49 | t=-0.86; p=0.39 |
| **Covariates** | | |
| Age | r=0.34; p=0.000* | r=-0.03; p=0.72 |
| GA at sample acquisition | r=0.20; p=0.02* | r= -0.17; p=0.06 |
| Maternal education | t= -3.04; p=0.003* | t= -1.41; p=0.16 |
| Insurance status | t= -2.39; p=0.02* | t=2.40; p=0.02* |
| BMI | r=0.13; p=0.13 | r=0.16; p=0.06 |
| Cigarette smoking | t=1.88; p=0.06 | t=0.95; p=0.35 |
| Marijuana Use | t=1.14; p=0.26 | t=1.22; p=0.22 |
| Parity | t=-1.62; p=0.11 | t=-0.04; p=0.96 |

[A] Kruskal Wallis non-parametric test used
[B] Fisher's Exact test used

*=statistically significant at p<0.05

Variables shaded in green included in the final regression models

**Table 4: Linear Regression Models: Community State Type with Complement Markers C3a and Bb**

| *Variable* | *B* | *p* | *95% CI* |
|---|---|---|---|
| [1] Assay plate | 1148.95<br>528.22<br>1498.35<br>2647.71<br>Reference | <0.001 | [Plate 1: 621.23, 1676.67]<br>[Plate 2: -202.37, 1258.80]<br>[Plate 3: 834.93, 2161.76]<br>[Plate 4: 2038.78, 3256.64]<br>[Plate5: Reference] |
| [2] Age | 75.83* | 0.02 | [26.50, 144.80] |
| CST | A | 0.62 | A |
| Education | -483.17 | 0.21 | [-1236, 270.09] |
| Insurance | -261.75 | 0.38 | [-853.53. 330.04] |
| Tobacco | 421.26 | 0.29 | [-377.52, 1220.04] |
| BMI | 15.42 | 0.34 | [-15.66, 46.49] |
| Ga Sample | 24.56 | 0.60 | [-67.79, 116.91] |

Model 1: F=9.69; p<0.001; $R^2$=0.52

Model 2: F=2.683; p=0.006; $R^2$=0.19

| *Variable* | *B* | *p* | *95% CI* |
|---|---|---|---|
| [3] CST | A | 0.97 | A |
| Insurance | 0.20 | 0.08 | [-.02, 0.43] |
| Tobacco | 0.20 | 0.24 | [-0.13, 0.53] |
| BMI | 0.01 | 0.06 | [0.00, 0.03] |
| Ga sample | -0.06 | 0.08 | [-0.10, 0.06] |
| | | | |
| [4] CST | A | 0.78 | A |
| Insurance | 0.11 | 0.50 | -0.21, 0.43 |
| Tobacco | 0.25 | 0.21 | -0.14, 0.64 |
| BMI | 0.01 | 0.21 | -0.01, 0.03 |
| Ga sample | -0.03 | 0.24 | -0.10, 0.03 |

Model 3: F=1.60; p=0.13; $R^2$=0.09

Model 4:F=1.14, p=0.35; $R^2$=0.11

*=statistically significant at p<0.05

Abbreviations: C3a, complement marker C3a; Bb, complement marker Bb; CST, community state type

[1,2] Interassay variability high for C3a during sample analysis. Model 1 shows the initial model with all samples included. Model 2 excludes women with variation >10% between duplicates

[A]Multiple Betas for each CST and each plate in are not shown due to space limitations however the p value is presented

[3,4] Interassay variability in acceptable range for Bb, however conducted similar quality control measures by excluding women with variation >10% between duplicates. Model 3 is the initial model with all samples included. Model 4 excludes women with >10% variation between duplicates

**Table 5: Linear Regression Models: Reproductive Tract Infection and Complement Markers C3a and Bb**

**C3a**

| *Variable* | *B* | *p* | *95% CI* |
|---|---|---|---|
| Reproductive Tract infection <20 weeks | -165.91 | 0.51 | [-662.02, 330.19] |
| Ga sample | 82.39 | 0.16 | [-31.75, 196.53] |
| BMI | 10.18 | 0.49 | [-18.72, 39.09] |
| Insurance | -161.21 | 0.57 | [-720.88, 398.47] |
| Education | -558.10 | 0.11 | [-1247.83, 131.62] |
| Age* | 80.90 | 0.006 | [24.48, 141.68] |
| Smoking | 527.55 | 0.17 | [-229.39, 1284.49] |

F=3.72; p=0.001; $R^2$=0.17

**Bb**

| *Variable* | *B* | *p* | *95% CI* |
|---|---|---|---|
| Reproductive Tract infection <20 weeks | -0.08 | 0.57 | [-0.36, 0.20] |
| Ga sample | -0.05 | 0.10 | [-0.12, 0.01] |
| BMI | 0.01 | 0.24 | [-0.01, 0.03] |
| Insurance | 0.15 | 0.38 | [--0.18, 0.48] |
| Education | -0.18 | 0.29 | [-0.52, 0.15] |
| Smoking | 0.23 | 0.24 | [-0.16, 0.62] |

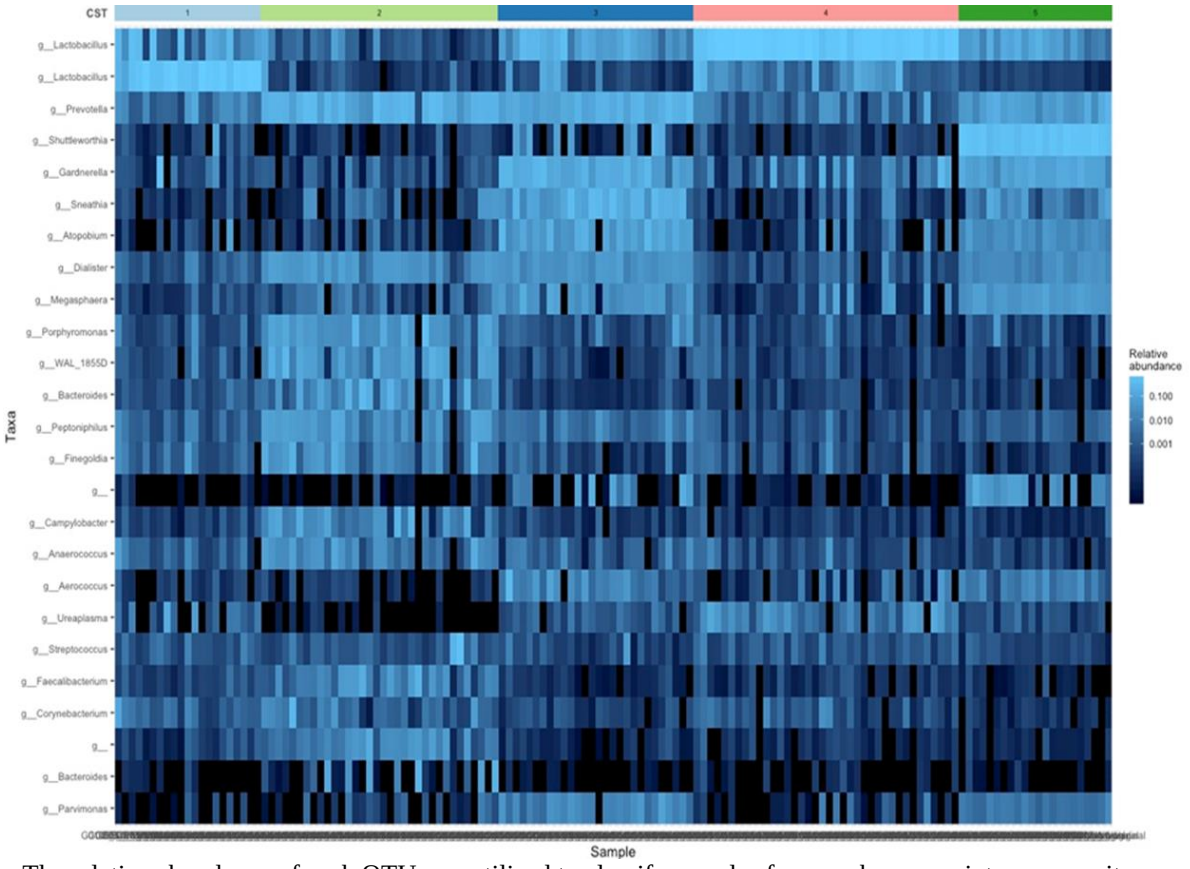
F=1.52; p=0.183; $R^2$=0.11

**=statistically significant at p<0.05

Abbreviations: C3a, complement marker C3a; Bb, complement marker Bb; BMI, body mass index

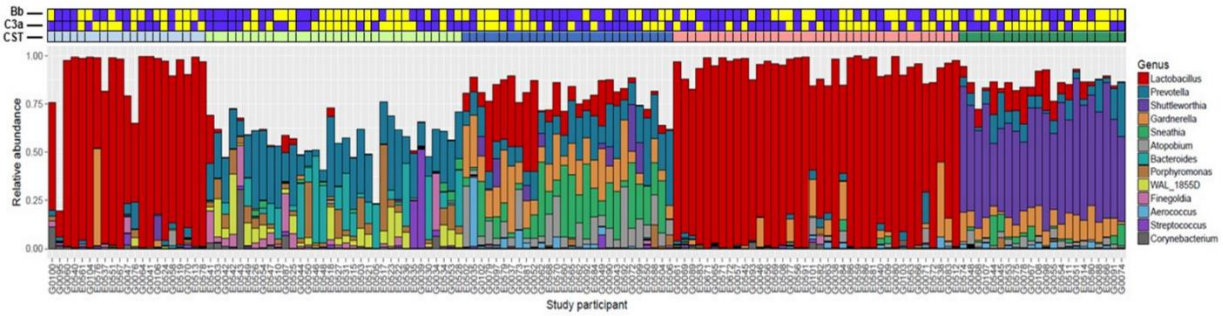
All analyses conducted for women with <10% variation between duplicates for complement markers

**Figure 1: Heat Map of Vaginal Microbiome Community State Types**




The relative abundance of each OTU was utilized to classify samples from each woman into community state types using the method previously described by DiGiulio et al. Samples were clustered into five community state types: (1) CST 1 had a high relative abundance of the traditionally healthy type *Lactobacillus*; (2) CST 2 had the lowest levels of *Lactobacillus* with a higher relative abundance of *Prevotella*, *Bacteroides*, and *Porphyromonas*; (3) CST 3 had a high relative abundance of *Sneathea* and *Gardnerella*; (4) CST 4 was dominated by *Lactobacillus iners*; (5) CST was dominated by *Shuttleworthia*.

**Figure 2: Community State Types and Complement Levels**




Heat map depicting the relative abundance of the vaginal microbiome for each study participant grouped together by community state type. The corresponding complement level is shown above each study participant. Women with C3a and Bb levels that fall above the median concentration are designated in yellow; women with C3a and Bb levels that fall below the median concentration are designated blue.

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# Chapter 4:

## Complement Activation During Early Pregnancy and Clinical Predictors of Preterm Birth in African American Women

## ABSTRACT

**Introduction**: Complement activation during pregnancy is essential for processes related to implantation, maternal-fetal immune tolerance, and the maternal response to infection. However, excess activation of complement has also been associated with adverse pregnancy outcomes including preterm birth (PTB). African American (AA) women experience a disproportionately higher rate of inflammation associated PTB compared to other groups of women, thus the purpose of this study is to confirm the contribution of complement activation to the development of adverse pregnancy outcomes in a high risk population. Specifically, this study explored whether elevations in 1$^{st}$ trimester concentrations of complement activation fragments C3a and Bb are associated with early pregnancy clinical risk factors for PTB as well as gestational age at delivery. The identification of clinical parameters that correlate with complement activation during early pregnancy may prove beneficial in the identification of women at highest risk for later onset of PTB.

**Methods**: A plasma sample was collected between 8-14 weeks gestation from a cohort of women (N=144) enrolled in a larger study investigating biobehavioral determinants of the microbiome and risk factors for PTB in African American women. The plasma samples were frozen at -80C until the time of complement assay analysis. Chart review was conducted to collect information on clinical factors associated with PTB (e.g., twenty-week cervical length, health behaviors) along with the gestational age at delivery.

Multivariable linear regression modeling was used to explore the relationships between concentrations of complement markers (C3a/Bb) and the select clinical risk factors.

**Results**: Linear regression analysis concluded that early pregnancy C3a and Bb concentrations are not associated with the gestational age at delivery or the twenty-week cervical length. Significant variables associated with the gestational age at delivery include age ($B$=0.21, p=0.03) and no history of reproductive tract infection diagnosed prior to 20 weeks gestation ($B$=-1.66, p=0.04). The only variable associated with the twenty week cervical length was a history of PTB ($B$=0.59, p=0.007). There were no differences in the mean concentration of C3a and Bb for select health behaviors.

**Discussion**: Findings from this study do not indicate a significant relationship between complement markers, gestational age at delivery, or the 18-20 week sonographic cervical length. There were also no associations found between complement and behavioral risk factors associated with PTB. The findings of this study suggest that markers of complement system activation do not influence pregnancy outcomes among AA women in the same way as predominantly white populations, however more studies are needed to define the true definition of complement system dysregulation and the mechanisms for adverse pregnancy outcomes among AA women.

**Keywords:** early pregnancy, complement system, inflammation, Preterm Birth

## INTRODUCTION

Preterm Birth (PTB, birth <37 weeks) is the leading cause of infant morbidity and mortality in the US, resulting in significant economic, emotional, and long-term health consequences for patients and families.[1] Annually 1 in 10 infants – approximately 380,000-- are born preterm, with a significant disparity noted in rates among minority groups, particularly African American women who are nearly twice as likely to experience preterm delivery than white women.[2] The underlying etiologic mechanisms underpinning PTB involve changes in both maternal and fetal biological systems, as well as local changes that affect the activities of the intrauterine environment.[3] Efforts to decrease the rates of PTB are challenging as the nature of the syndrome is multifactorial involving factors such as stress [4,5], systemic and intrauterine infection and inflammation, [6-8] uterine overdistention, [3,9] endocrine disorders, [10] and cervical dysfunction.[11-13]

As such, recent PTB studies have focused on the identification of early pregnancy biobehavioral pathways associated with changes in the intrauterine and cervical environment. One pathway strongly associated with increased PTB risk is intrauterine infection and inflammation.[3,11,14] Interestingly, inflammation associated PTB is thought to be one of the leading biological pathways to PTB among African American (AA) women as supported by their elevated rates of PTB<34 weeks and elevated rates of preterm premature rupture of membranes (PPROM), which are more likely to occur in the presence of infection.[15] The inflammatory processes associated with infection trigger the activation of several immune pathways, which are thought to promote premature activation of the myometrium and cervical remodeling via the production of potent

inflammatory mediators including pro-inflammatory cytokines, matrix metalloproteinases, prostaglandins, and complement proteins.[16-18]

Randomized controlled trials targeting the elimination of risk factors such as infection have not been effective in decreasing the incidence of PTB.[19] Sexual and vaginal hygiene practices (e.g. douching, multiple sex partners), although not previously shown to have a direct association with an increased PTB risk, may potentially interrupt vaginal protective mechanisms (e.g. ph, lactobacilli, hydrogen peroxide), thereby promoting the growth of microbes that could lead to infection.[20] Sexual health behaviors may also promote changes that alter the integrity of the vaginal and rectal tissues, making the opportunity for infection more favorable. Exploring these health behaviors as they relate to inflammatory processes may prove beneficial in the identification of women at highest risk for infection and possibly PTB.

Recently, complement associated immune and inflammatory events in early pregnancy have been identified as predictive biological factors in the development of adverse pregnancy outcomes.[18,21,22] Specifically, one study of predominantly Caucasian women (7%) found that women who had C3a levels in the upper quartile were nearly three times as likely to have an adverse pregnancy outcome compared to women with C3a levels in the lowest quartile; associations were primarily related to hypertensive disorders of pregnancy, PTB, and preterm premature rupture of membranes. [18] Similarly another study, also of predominantly Caucasian women (5%) found that women with elevated levels of the complement fragment Bb during early pregnancy were almost four times as likely to have later onset of spontaneous PTB when compared to women with complement fragment Bb levels in the lower three quartiles.[21] The biochemical

processes associated with inflammation are thought to stimulate changes that promote uterine contractility, enhance cervical ripening, and increase decidual activation,[3,9] thereby increasing the risk for PTB.

Therefore, the purpose of this study was to confirm the contribution of complement activation to the development of select pregnancy outcomes in a high risk population and to explore the relationship between early pregnancy concentrations of the complement biomarkers (C3a and Bb) and clinical risk factors associated with PTB in AA women: gestational age at delivery, mid pregnancy cervical length, and select health behaviors (e.g. smoking, sexual risk behaviors). There were two hypotheses tested; first that women with higher concentrations of complement plasma levels of C3a and Bb would have shorter cervical lengths at 20 weeks and/or a shorter gestational age at delivery, and second that women with health behaviors previously associated with PTB would demonstrate higher concentrations of C3a or Bb. To our knowledge, no previous studies have reported a relationship between complement concentrations and clinical risk factors for PTB. In this paper, we report those findings and discuss the importance of such findings within a clinical context.

## METHODS

### Sample and Setting

Pregnant African American (AA) women were recruited during their 1st trimester of pregnancy via outreach to local prenatal clinics associated with a public and a private hospital in Atlanta Georgia. Women were asked at check-in if they would be interested in learning about an on-going study of the "Microbiome and Biobehavioral Risk Factors for Preterm Birth in AA Women." After an initial screening, those who indicated willingness

to participate in the study, and who met inclusion criteria, received a full description of the study, and were asked to provide informed consent; those who did were then enrolled. Women next completed a series of demographic, stress, dietary, and behavioral questionnaires including questions related to vaginal hygiene practices, sexual practices, infection history, and other health behaviors experienced in the last month after which they were accompanied to their prenatal blood draw, where an extra sample of blood (30ccs) was drawn for later measurement of immune and inflammatory variables. Data and samples were collected from a subset of 144 women who had plasma samples available for analysis of 1$^{st}$ trimester complement biomarker plasma levels of C3a and Bb. Additionally, a more detailed review of their medical records post-delivery including identification of clinical risk factors associated with PTB as well as a clinical measure of 2$^{nd}$ trimester cervical length was collected post delivery. These variables were evaluated in light of participant self-report of biobehavioral risk factors related to infection and sociodemographic factors. Given that SES is a determinant of many health behaviors, the diversity across the two hospitals provided a diverse group of women in which to explore complement biomarkers and clinical risk factors for PTB. The study was approved by the Emory Institutional Review Board.

**Inclusion/Exclusion Criteria**

Prenatal inclusion criteria were that women identify as AA race (via self report), which allowed for an in-depth analysis of intra-race risk factors for complement activation. This design is consistent with recommended frameworks for studying racial disparities which recommend exploring risk and protective factors within the high risk

group. [23] Additional criteria included: singleton pregnancy between 8-14 weeks gestation as verified by clinical record, able to comprehend written and spoken English, and free of chronic illness or medication use including use of anti-inflammatory agents or antibiotics at the time of enrollment. Additionally, women were excluded postnatally if there was a fetal death prior to labor or documentation of congenital anomalies. Details regarding the full inclusion exclusion criteria and enrollment process are published elsewhere (Corwin, 2017).

**Measures: Clinical Questionnaires**

*Prenatal Health Survey*

A 27-item prenatal health survey, a measure utilized within the parent study protocol, was administered to women between 8-14 weeks to ascertain self-report of infection history, medication use, sexual history (e.g. type of intercourse, number of partners, and use of condoms), hygiene self-care practices (e.g. douching, vaginal sprays), and substance use (e.g. smoking or illegal drug use) within the previous month. Questions such as "Have you had any infections of your vagina" were answered with "yes" or "no." If the woman answered "yes" additional questions followed regarding the time frame of participation in the activity ("When were you diagnosed with this vaginal infection"), and details regarding the use of additional supplements, prescriptions, or over the counter medications.

To protect the mental health of a study participant, the interviewer engaged in an open discussion of the potential mental stress related to answering questions regarding sexual history, vaginal hygiene practices, and substance use. She emphasized that the respondent did not have to answer any question that made her uncomfortable.

Furthermore, all interviewers were trained to recognize signs of psychological distress and followed a protocol of contacting the woman's primary care provider, or other supportive provider, should she show signs of distress from any of the questions. One of the instruments was the Edinburgh Depression Scale, and any woman who scored > 12 was referred for mental health counseling.

*Sociodemographics Questionnaire*

A 10-item self-report sociodemographics questionnaire was administered to women between 8-14 weeks to collect information regarding family size, income, age, education level, relationship status, and insurance status. Additionally, chart review was conducted post-delivery to confirm info regarding any changes in sociodemographic factors such as relationship or insurance status.

**Measures: Clinical Variables**

Maternal medical chart abstraction was completed using a standardized abstraction tool[24] following delivery to abstract clinical variables including the twenty-week cervical length, pre pregnancy body mass index (BMI), reproductive tract infection prior to 20 weeks, and other information about the intrapartum course (e.g. gestational age at delivery). Information about the twenty-week cervical length was collected from the anatomy ultrasound conducted between 18 and 20 weeks. Cervical length measures were evaluated for normalcy based on current clinical guidelines which define a shortened cervical length as 2.5cm or less.[25] Pre-pregnancy BMI was calculated via measurement of the height and weight at the first prenatal visit and categorized according to Institute of Medicine guidelines (obesity $\geq$ 30 kg/m$^2$, overweight 25-29.99 kg/m$^2$, healthy weight 18.5-24.99 kg/m$^2$, and underweight <18.5 kg/m$^2$). Clinical diagnoses of

infection were confirmed via review of the clinical record and/or evaluation of laboratory results. Data regarding intrapartum course and gestational age at delivery were collected according to questions on the abstraction tool; gestational age was defined as the gestational week at the time of delivery as confirmed by LMP or first trimester ultrasound.

**Measures: Complement Biomarkers**

*C3a and Bb*

Plasma levels of the complement fragments C3a and Bb were measured from blood samples collected during the first visit of the larger study. C3a levels were measured using an enzyme-linked immunosorbent assay (ELISA) kit (BD Opt EIA, BD Biosciences, CA), and Bb levels were measured with MicroVue Bb Plus Fragment EIA kit (Quidel Corporation, OH). Each were processed according to the manufacturer's recommendations. The absorbance readings were measured using a Synergy 4 Microplate Reader (BioTek, VT) at 450 nm. Gen5 curve-fitting statistical software (BioTek, VT) was employed for calculation of ELISA results. The inter-assay (plate to plate) coefficients of variation (CV%) were 18.1 for C3a and 5.3 for Bb; the intraassay (within plate) coefficients of variation were 5.8 for C3a and 17.6 for Bb.

**Statistical Analysis**

Data were analyzed using both descriptive and inferential test statistics. To identify distributions, outliers, and missing data, analysis included measures of central tendency and dispersion to examine and compare the frequencies, means, and variability measures for select variables. Relationships between categorical variables were evaluated with the Chi-square test or Fisher's exact test. Relationships with continuous variables

were evaluated using Pearson correlations and Student t-tests. Additional exploratory analyses were done to explore the differences in the mean concentrations of C3a and Bb for a range of health behaviors associated with an increased risk of infections and/or PTB (e.g. smoking, marijuana use, douching). Controlling for select covariates, multivariable linear regression was used to explore the relationship between C3a and Bb with the mid pregnancy cervical length and gestational age at delivery. An additional analysis was done to explore how these factors that influence the gestational age at delivery for women with spontaneous labor only. The data were analyzed in SPSS 24.0. Statistical tests were two-sided with a p-value<0.05 used to determine statistical significance.

## RESULTS

### Descriptive Statistics

A total of 144 African American women were enrolled in the study. Women were relatively young (median age 23.5±6.0), primarily Medicaid insured (70.8%); 54% were educated at the college level or higher. A majority of the women were single, although 50% were involved in relationships in which they cohabitated with a partner (Table 1). The majority of women in the cohort had a spontaneous labor onset (55.6%), although 15 (10.4%) had no labor, and 46 (31.9%) were induced. The median gestational age at delivery was 39.2 weeks, with a PTB rate of 29% (n=20). The mean cervical length was 3.8±0.8 cm, however only 3.5% of the cohort had a diagnosed shortened cervix (cervical length <2.5cm), which is a strong clinical predictor of PTB.[25,26] The mean concentration of complement marker C3a was 4001.43±118.77 ng/mL and 1.48±0.05 μg/mL for Bb.

A range of health behaviors including substance use, sexual history and vaginal hygiene practices during early pregnancy are presented in Table 2. 9.7% reported tobacco use and 14.6% reported marijuana use during early pregnancy. Many women in the cohort engaged in vaginal intercourse (n=113), with the majority of women reporting only one sexual partner in the last month (n=105). Of the 113 women who reported having vaginal intercourse, 110 reported engaging in intercourse with no condom use. 37% of the cohort reported engaging in vaginal and oral intercourse, but only 2% had anal intercourse. There were no women in the sample that reported other types of intercourse who were not also having vaginal sex. A small number of women engaged in various hygiene practices including douching (n=6), use of feminine sprays (n=20) and wipes, and various creams and home remedies (n=4).

**Univariate Associations**

Associations between the independent variables (C3a and Bb), covariates (e.g. age, education level), and dependent variables (twenty-week cervical length and gestational age at delivery) are outlined in Table 3. There are statistically significant associations between age (p=0.04), history of PTB (p=0.009), insurance status (p=0.03) and the twenty-week cervical length measurement. As age increases, the cervical length increases (r=0.20, p=0.04), however in women with a history of PTB, the mean cervical length is shorter (3.2±0.79 cm) compared to women with no previous history (3.9±0.73 cm). Additionally, women insured by Medicaid had a shorter mean cervical length (3.67±0.64 cm) compared to women who were privately insured (4.03±0.93 cm). There were no significant associations between select variables and the gestational age at delivery, although several factors that have been previously associated with PTB were

evaluated and trended toward a contribution, including a history of PTB (p=0.06), insurance status (p-0.08), body mass index (p=0.09), and early pregnancy reproductive tract infection (p=0.09). Student t-tests were done to explore whether there were differences in the mean concentration of C3a and Bb for the various health behaviors, however there were no significant relationships identified (Table 4).

**Regression Analyses**

*Cervical Length*

Controlling for select covariates, there was a significant linear relationship identified between cervical length and the covariates taken together (F=3.65, p=0.003, $R^2$=0.19), however C3a (p=0.41) and Bb (p=0.23) were not significant variables in the model. The only significant independent variable identified was having a history of PTB (p=0.007). For a woman with no prior history of PTB, the cervical length measurement would be expected to be higher by 0.59cm compared to a woman with a previous history (Table 5). A second analysis was done to explore the relationship between C3a and cervical length if a history of PTB was removed; the results indicated that 12.5% of the variance in the cervical length measurement could be attributed to the early pregnancy BMI (F=2.69, p=0.03, $R^2$=0.125). For every 1 unit increase in BMI, the cervical length would be expected to increase by 0.02cm (β=0.02; p=0.04).

*Gestational Age at Delivery*

Linear regression analysis indicated a significant linear relationship between gestational age at delivery and the covariates taken together (F=2.42, p=0.02, $R^2$=0.11), however C3a (p=0.21) and Bb (p=0.54) were not significant variables in the model. Age (*B*=0.21, p=0.03) and no history of reproductive tract infection <20 weeks (*B*=-1.66,

p=0.04) were the only significant variables. For every one year increase in age, the gestational age is expected to increase by 0.2 weeks (1.4 days). The gestational age at delivery was also influenced by the history of reproductive tract infection prior to 20 weeks in the linear regression analysis. It was shown that women who did not have a reproductive tract infection prior to 20 weeks would have a gestational age at delivery 1.6 weeks less than someone who had an early pregnancy infection (table 6).

A second analysis was done to explore factors related to the gestational age at delivery among women with spontaneous labors only. There was not a significant linear relationship identified between gestational age and the covariates taken together ($F=1.26$, $p=0.28$; $R^2=0.11$), although a history of PTB was significant variable in the model ($B=2.84$, $p=0.03$). Women with no history of PTB would be expected to have a gestational age at delivery 2.8 weeks longer than someone with a history of PTB.

## DISCUSSION

The purpose of this study was to explore the contribution of complement activation to the development of select pregnancy outcomes (e.g. cervical length and gestational age at delivery) in a high risk population, as well as to examine the relationship between complement activation and select health behaviors previously associated with PTB. Our findings do not indicate any significant relationships between complement, the twenty-week cervical length, or the gestational age at delivery. The identification of other mechanisms that may influence cervical length or gestational age at delivery are worth investigating, as findings from this study suggest that markers of complement system activation does not appear to have a significant influence on these

outcomes among AA women. Additionally, no relationships were identified between the complement markers and behavioral variables of interest (Table 4).

The linear regression analyses found that age appears to be an important factor as it relates to the gestational age a delivery. For every one year increase in age, the gestational age is expected to increase by 0.2 weeks/1.4 days, suggesting that advancing age may be protective against early delivery. Some studies have shown that women who are very young as well as women over the age of 35, have an increased risk for pregnancy complications including genetic defects, gestational diabetes, pregnancy associated hypertension, and PTB.[27,28]. Interestingly the association between age and risk of PTB varies according to race.[29] PTB rates have been shown to rise at a younger age for AA as compared to white women, which according to Geronimus is attributed to weathering. She proposes that social injustice compounds with age leading to health inequity for AA that influences reproductive health outcomes.[29] None of the women in this sample were older than 35. More studies are needed to understand how age and ethnicity influences PTB rates.

The gestational age at delivery was also influenced by the history of reproductive tract infection prior to 20 weeks in the linear regression analysis. It was shown that women who did not have a reproductive tract infection prior to 20 weeks would have a gestational age at delivery 1.6 weeks less than someone who had an early pregnancy infection. This finding is opposite of what is found in the literature, which suggests that reproductive tract infection is a risk factor for PTB, particularly among AA women.[15] Infection associated preterm birth among AA has been linked to an increased prevalence of lower genital tract infection in other studies,[6,15] however in this group it appears that

women who had a reproductive tract infection prior to twenty weeks had longer gestation compared to women who did not. This relationship was further explored using a student t-test to see if there was difference in the mean gestational age between women with and without infection. There were not significant differences in the mean gestational age between the groups (t=-1.40, p=0.16). The mean gestational for someone with an infection was 38.7 ±2.6 compared to someone with no infection 37.6 ±5.1.

The complement markers were not associated with the clinical outcomes in this study. Early pregnancy complement -associated inflammation has been associated with an increased risk for PTB in previous studies, however these complement related PTB studies have been conducted in primarily white populations, a group at low risk for inflammation-driven PTB. It is possible that C3a and Bb elevation in AA women may not have the same physiologic consequences or increased risk for pregnancy complications as shown in other populations.

Sonographic cervical length is a tool commonly used to assess for PTB risk; women with a shortened cervical length at 20 weeks have an increased risk for spontaneous PTB.[13] Traditionally the cervical length has been evaluated only as an anatomic structure;[13] however newer studies suggest that the cervical length should be evaluated as both an anatomic and biological contributor by exploring the cervix in relation to biochemical pathways associated with labor onset.[13] The identification of biological mechanisms that influence both term and preterm cervical remodeling may prove beneficial in understanding the risk for PTB among high risk group groups. Unfortunately, the findings of this study do not show a relationship between markers of

complement system activation and cervical length. The most significant factor associated with the twenty-week cervical length in this study was having a previous history of PTB.

**Limitations**

One of the significant predictors of having an earlier gestational age at delivery was having no history of a reproductive tract infection prior to twenty weeks gestation; specifically, women with no history of a reproductive tract infection would be expected to deliver 1.6 weeks sooner than a woman with an infection. This is an odd finding as infection is a known risk factor for earlier delivery.[6,30] The reproductive tract infection variable in the data set is a variable that takes into account several different types of reproductive tract infections including bacterial vaginosis, urinary tract infection, chlamydia, trichomoniasis, gonorrhea, and candidiasis; the better option may be to tease out each type of infection and looking at the individual relationships with gestational age as this may be more meaningful and reflective of findings in the literature. This was not an option in this particular analysis as the sample size for each type of infection was too small to look at individually in the regression models. Future analyses should also consider clustering different types of infections together. For instance, some types of vaginal conditions are not an "itis" but an "osis." Vaginosis implies that there is infection in the vagina, whereas other conditions such as vaginitis implies that there is inflammation in the vaginal environment, which could result from many factors including allergies, irritants, or hormonal changes. Carefully selecting how infections are grouped together may result in findings more reflective of the literature.

Secondly it was found that the complement biomarkers were not associated with the select pregnancy outcomes. This finding may be a result of the strict nature of the larger study inclusion/exclusion criteria which may have eliminated the women who are more likely to have adverse pregnancy outcomes and/or excessive complement activation, such as women with chronic medical conditions or other risk factors for exaggerated inflammation. Including high risk pregnant women with chronic conditions in research studies is challenging, as pregnant women represent a vulnerable population; nevertheless, these strict criteria may influence the ability to detect mechanisms associated with adverse outcomes simply because the women enrolled into the study may not be representative of the populations at highest risk for poor outcomes.

This study measured markers of complement system activation, but it is unknown if the concentrations observed in this population represent true "complement dysfunction." In the literature complement dysregulation has been defined as excessive activation or inadequate regulation,[18] however this study is limited by the fact that only two markers of complement activation were explored. Future studies should consider including other mechanisms of complement system activity includes measures of not only complement activation fragments, but also other effector mechanisms of the complement pathway including other anaphylatoxins (ex C5a), opsonins (C3b, C3d), complement regulatory proteins (e.g. Factor H, CR1) and markers of the membrane attack complex (C5b-C9). Effective interplay between these factors ensures appropriate complement system function, which results in a successful defense against bacteria and well controlled inflammation due to tight control of inflammatory mediators. For these

reasons, the complement system should not be ruled out as a contributor to adverse outcomes in AA women.

## CONCLUSIONS

The prolonged and/or premature activation of pro-inflammatory immune factors (e.g. cytokines, prostaglandins, complement) are thought to transform the myometrium from a quiescent to contractile environment, promote changes that decrease the tensile strength of the cervix, and weaken the amniotic membranes which have been implicated as key biological risk factors in the etiology of PTB.[21,31] Dysregulation in the activities of the complement pathway have been implicated in a variety of adverse pregnancy outcomes including PTB, however the mechanisms are not fully understood. [18,21,22,32] The findings of this study suggest that markers of complement system activation do not influence pregnancy outcomes among AA women in the same way as predominantly white populations, however more studies are needed to define the true definition of complement system dysregulation and the mechanisms for adverse pregnancy outcomes among AA women.

## Tables and Figures

**Table 1.** Demographic and Clinical Characteristics of the Cohort (N=144)

| Sociodemographics | Mean (SD), Median (IQR), or n (%) |
|---|---|
| **Age, median (IQR)** | 23.5 (6.0) |
| **Insurance Status, n (%)** | |
| Medicaid | 102 (70.8) |
| Private | 42 (29.2) |
| **Race, n (%)** | 144 (100) |
| African American | |
| **Education Level, n (%)** | |
| High School or less | 66 (45.8) |
| College or greater | 78 (54.2) |
| **Marital Status, n (%)** | |
| Married | 19 (13.2) |
| Single | 125 (86.8) |
| **Relationship Status, n (%)** | |
| not in a relationship | 28 (19.4) |
| in a relationship (no cohabitation) | 43 (29.9) |
| in a relationship (cohabitation) | 73 (50.7) |

Data are presented as mean ±standard deviation or standard error, median (IQR), or n (%)

**Table 2: Complement Measures, Clinical Outcomes, and Health Behaviors (N=144)**

| Clinical Variables | Total Cohort[AB] |
|---|---|
| **C3a (ng/mL), mean ±SE (median)** | 4001.43± 118.77 (3781.51) |
| **Bb (µg/mL), mean ±SE (median)** | 1.48± 0.05 (1.37) |
| **BMI at 1st prenatal visit, median (IQR)** | 26.1 (10.9) |
| **Cervical Length (cm), mean (SD)** | 3.8 (0.8) |
| **Cervical Length less than 2.5cm, n (%)** | 5 (3.5) |
| **Prior Cervical Procedure (LEEP, Conization)** | 1 (0.7) |
| **Type of Labor, n (%)**<br>None<br>Spontaneous<br>Induced | <br>15 (10.4)<br>80 (55.6)<br>46 (31.9) |
| **Preterm Birth (PTB), n (%)**<br>Yes<br>No | <br>20 (13.9)<br>124 (86.1) |
| **Gestational age at Delivery, median (IQR)** | 39.2 (1.7) |
| | |
| **1st Trimester Health Behaviors** | **Total Cohort[C]** |
| **Smoking, n (%)** | 14 (9.7) |
| **Marijuana use, n (%)** | 21 (14.6) |
| **Number of Vaginal Sex Partners, n (%)**<br>**1**<br>**2** | <br>105 (72.9)<br>1 (0.7) |
| **Any Vaginal Intercourse, n (%)**<br>No<br>Yes | <br>27 (18.8)<br>113 (78.5) |
| **Type of Vaginal Intercourse**<br>Vaginal only (with condom)<br>Vaginal only (no condom)<br>Vaginal + oral intercourse<br>Vaginal + anal intercourse | <br>3 (2.1)<br>110 (76.4)<br>52 (37.4%)<br>3 (2.2) |

| | |
|---|---|
| **Hygiene Self-Care Practices, n (%)** | |
| Douching | 6 (4.2) |
| Feminine Sprays/Wipes | 20 (13.9) |
| Creams/Home Remedies | 4(2.8) |

[A]Data are presented as mean ±standard deviation or standard error, median (IQR), or n(%)

[B] Cervical length n=100

[C] Smoking n=140; marijuana use n=139; sexual partners n=106, vaginal sex n=140; anal sex n=139; oral sex n=139; condom use n=103; douching n=139; sprays/wipes n=138; creams/home remedies n=139

all health behaviors assessed at enrollment and address activities within the past 30 days

**Table 3: Associations Between Independent Variables, Covariates, and Dependent Variables**

| IV/Covariates | 18-20 Week Cervical Length | Gestational Age at Delivery |
|---|---|---|
| C3a | r=-0.01; p=0.95 | r=0.01; p=0.88 |
| Bb | r=-0.16; p=0.12 | r=0.04; p=0.68 |
| Age | r=0.20; p=0.04* | r=0.17; p=0.05 |
| Maternal education | t=-0.32; p=0.75 | t=-0.21; p=0.83 |
| History of Preterm Birth | t=2.67; p=0.009* | t=1.85; p=0.06 |
| Insurance status | t=-2.01; p=0.03* | t=-1.76; p=0.08 |
| BMI | r=0.20; p=0.05 | r=0.14; p=0.09 |
| Cigarette smoking | t=0.74; p=0.46 | t=0.70; p=0.48 |
| Marijuana Use | t=0.60; p=0.55 | t=0.57; p=0.57 |
| Reproductive Tract Infection <20 weeks | t=0.57; p=0.57 | t=-1.69; p=0.09 |
| Antibiotic use | t=0.71; p=0.48 | t=-0.86; p=0.39 |

*=statistically significant at p<0.05

**Table 4: Complement Levels and Select Health Behaviors**

| Health Behaviors | C3a | Bb |
|---|---|---|
| **Smoking** | t=1.88; p=0.06 | t=0.95 p=0.35 |
| **Marijuana use** | t=1.14; p=0.26 | t=1.22; p=0.22 |
| **More than one sexual partner** [A] | - | - |
| **Vaginal Intercourse** | t=0.21; p=0.83 | t=0.95; p=0.35 |
| **Type of Intercourse** | | |
| Vaginal only (with condom) | t= 0.92; p=0.36 | t= -0.85; p=0.40 |
| Vaginal only (no condom) | t= -0.12; p=0.91 | t= 1.12; p=0.23 |
| **Hygiene Self-Care Practices** | | |
| Douching | t=1.20; p=0.23 | t= -0.36; p=0.72 |
| Feminine Sprays/Wipes | t=0.99; p=0.33 | t=0.07; p=0.95 |
| Creams/Home Remedies | t= -0.48; p=0.63 | t=0.14; p=0.89 |

*=statistically significant at $p<0.05$

[A] only one woman reported having more than one sexual partner

**Table 5: Linear Regression Models of Complement and Cervical Length**

| *Variable* | *B* | *p* | *95% CI* |
|---|---|---|---|
| C3a | 0.00005 | 0.41 | [0.00, 0.007] |
| Bb | -0.15 | 0.23 | [-0.39, 0.09] |
| No Hx of Preterm Birth *[A] | 0.59 | 0.007 | [0.16, 1.01] |
| Insurance [B] | -0.24 | 0.16 | [-0.58, 0.09] |
| Age | 0.03 | 0.11 | [-0.01. 0.07] |
| BMI | 0.02 | 0.06 | [-0.01, 0.04] |

$F=3.65$ $p=0.003$ $R^2=0.19$

**=statistically significant at $p<0.05$

Abbreviations: PTB, Preterm Birth; C3a, complement marker C3a; Bb, complement marker Bb; BMI, body mass index

[A] Beta coefficient represents women coded 0 in the data set (0=no hx of PTB; 1=history of PTB)

[B] Beta coefficient represents women coded 0 in the data set (0=Medicaid insured; 2=private insurance)

**Table 6: Linear Regression Model: Complement and Gestational Age at Delivery**

| *Variable* | *B* | *p* | *95% CI* |
|---|---|---|---|
| [1]C3a | 0.00 | 0.21 | [-0.01. 0.00] |
| Bb | 0.40 | 0.54 | [-0.87, 1.67] |
| Hx of Preterm Birth[A] | 2.43 | 0.05 | [-0.02, 4.88] |
| Insurance[B] | -1.16 | 0.20 | [-2.96, 0.63] |
| Age | 0.21 | 0.03* | [0.02, 0.40] |
| BMI | 0.06 | 0.20 | [-0.04, 0.16] |
| Reproductive Tract Infection < 20 weeks (No) | -1.66 | 0.04* | [-3.27, -0.05] |
| [2]C3a | 0.00 | 0.21 | [-0.01,0.00] |
| Bb | 0.53 | 0.40 | [-0.72, 1.77] |
| Hx of Preterm Birth [A] | 2.840 | 0.03* | [0.23. 5.45] |
| Insurance[B] | -0.90 | 0.36 | [-2.86. 1.06] |
| Age | 0.12 | 0.22 | [-0.07, 0.31] |
| Reproductive Tract Infection <20 weeks (No) | -0.57 | 0.48 | [-2.16, 1.02] |

Model 1: F=2.42, p=0.02, $R^2$=0.11

Model 2 (Spontaneous Labor): F=1.26, p=0.28, $R^2$=0.11

*=statistically significant at p<0.05

Abbreviations: PTB, Preterm Birth; C3a, complement marker C3a; Bb, complement marker Bb; BMI, body mass index

Model 1: All women in the sample; Model 2: only women with spontaneous labor

[A] Beta coefficient represents women with no previous history of PTB

[B] Beta coefficient represents women with Medicaid insurance

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## Chapter 5: Discussion and Conclusion

The purpose of this dissertation was to explore the relationship between early pregnancy complement activation, the vaginal microbiome, and known biobehavioral risk factors for preterm birth (PTB) in African American (AA) women. The specific aims of this study were to: (1) Explore the relationship between the vaginal microbiome, reproductive tract infection, and complement C3a and Bb levels during early pregnancy; and (2) Investigate the relationship between early pregnancy complement C3a and Bb levels, the twenty-week mid pregnancy cervical length measurement, and the gestational age at delivery. Additionally, there was an exploratory aim to investigate the relationships between health behaviors associated with preterm birth (PTB) and complement C3a and Bb levels during early pregnancy.

Preterm birth (PTB), defined as birth prior to 37 weeks' gestational age, is a complication of pregnancy that is multifactorial and syndromic in nature; however, there is strong evidence that dysregulation in the activities of the immune system may be a key risk factor contributing to the underlying pathology of the syndrome. [1,2] One such mechanism recently reported to have an association with PTB is the complement system, a component of the innate immune response involved in the first line innate defense against pathogens.[3,4] Innate and adaptive immune mechanisms evolve during pregnancy to promote survival of the semi-allogenic fetus while simultaneously protecting the mother from attack by foreign invaders. [5] The complement system, functions at the implantation site to ensure successful trophoblast invasion and embryo implantation, both critical processes in normal placental and fetal development. [6] The complement system also enhances the activities of antibodies and phagocytic cells (e.g. macrophages) in the

process of eliminating bacteria and damaged cells from the body.[7,8] Upon activation complement proteins are cleaved resulting in inflammation, attraction of phagocytic cells, and formation of the membrane attack complex (MAC), thereby eliminating foreign and/or damaged cells. [9]

The complement system has a variety of triggers for activation, however dysregulation of the system may result from factors that promote excess activation and/or poor regulation, thereby promoting a heightened inflammatory state and potential attack to self-tissues. Although the complement system is necessary for normal physiologic function, two recent studies have identified elevated 1st trimester complement activation fragment C3a as a significant predictor of spontaneous PTB and preterm premature rupture of membranes, one of which was in a mouse model. [3,4] An earlier study of 1st trimester complement fragment Bb also reported that women with levels of complement fragment Bb in the top quartile were 4.7 times as likely to have a spontaneous PTB less than 34 weeks gestation compared to women with Bb in the lower quartiles. [10] A limitation of the human studies includes their racial homogeneity: only 7% of the women in the study of C3a and 5% of women in the study of Bb were African American—the group at greatest risk of PTB due to an infectious/inflammatory process.[11] AA women are 1.5 times as likely to experience PTB and nearly 2 times as likely to have an early PTB <32 weeks compared to Caucasian women.[12] This study sought to explore the contribution of complement system activation to select pregnancy outcomes among the group at highest risk for PTB, AA women.

## Summary of Research Findings

Each of the three papers (Chapters 2-4) included in this dissertation document adds a unique contribution to existing literature as it relates to the role of the complement system during pregnancy in AA women. Chapter 2 presents an in-depth literature review and mechanistic model of inflammation-associated PTB, which hypothesizes a relationship between the microbiome and dysregulation of the complement system. Chapter 3 presents findings related to Aim 1, which explored the relationships between the vaginal microbiome, infection, and complement C3a and Bb levels. Chapter 4 presents findings related to both Aim 2 and the exploratory Aim, which explored the relationships between early pregnancy C3a and Bb levels, the twenty-week cervical length, select health behaviors (e.g. smoking, sexual risk behaviors), and the gestational age at delivery. Chapters 2, 3, and 4 are discussed in more detail below.

### Chapter 2

The first manuscript (Chapter 2) summarizes the current knowledge regarding the role of infection and inflammation in the context of PTB, with a specific focus on the role of the microbiome and the complement system. The article presents a mechanistic model of inflammation-associated PTB, which hypothesizes a relationship between the microbiome and dysregulation of the complement system. The microbiome-complement activation pathway to PTB posits that pathogenic microbes from various locations (e.g., vaginal, oral, respiratory, gut, placental) influence the activation of complement pathways (e.g., C3a and Bb) in the maternal vasculature and reproductive tissues. The resulting inflammatory response involves the production of various inflammatory mediators, which

if dysregulated may break down collagen, stimulate uterine contractions, facilitate cervical ripening, and promote decidual activation, thereby increasing the risk for PTB.[13] Additionally, the model proposes that dysregulation of maternal complement activation at any point across gestation (implantation-37 weeks gestation) may increase the risk of PTB. The infection associated pathway to preterm birth is complex and may be influenced by a variety of factors related not only to the microbiome, but also the individual woman's inflammatory – and complement -- response to these factors.

**Chapter 3**

The second manuscript of this dissertation study (chapter 3), presents findings from the analysis of aim 1, which sought to explore the activities of the complement system in relation to early pregnancy reproductive tract infection and the vaginal microbiome. Analyses were done to explore the relationship between the vaginal microbiome classified by community state type (CST), clinically diagnosed reproductive tract infection (i.e. bacterial vaginosis, chlamydia, gonorrhea, urinary tract infection, and trichomoniasis), and C3a/Bb levels. The study hypotheses for Aim 1 were that: (1) women with CSTs dominated by primarily pathogenic bacteria would demonstrate higher levels of circulating C3a/Bb compared to women with CSTs dominated by healthier bacteria; and (2) women with reproductive tract infection during early pregnancy would demonstrate higher circulating C3a/Bb levels.

The microbial community of the vagina is diverse and consists of more than 50 species of bacteria that vary between women depending on host and environmental factors. [13,14] The vaginal microbial communities in this study were classified according

to the relative abundance of select microbes present and then grouped together by similarity into CSTs. The vaginal microbiome composition of women in the sample clustered into five distinct CSTs, each dominated by one of the following microbes: (1) CST 1 *Lactobacillus*; (2) CST 2 *Prevotella/Bacteroides*; (3) CST 3 Snethia/Gardnerella; (4) CST 4 *Lactobacillus iners*; (5) CST 5 *Shuttleworthia*.

Univariate associations between the CSTs and other covariates (i.e. age, education level) were explored; there were no significant relationships identified between select variables and the CSTs. In the final linear regression analyses, after controlling for select covariates (e.g. age, education level, smoking), there were no relationships identified between the vaginal microbiome CSTs and Bb levels ($F=1.60$, $p=0.13$, $R^2=0.09$). There was a significant relationship identified in the model of CST and C3a ($F=9.69$, $p<0.001$, $R^2=0.52$) however, the only significant variable identified in the initial model was the assay plate variable, which was included to control for the high interassay (plate to plate) variability that occurred during C3a assay analysis. C3a regression analyses were repeated using women who had less than 10% variation in concentration between the sample duplicates; there remained a significant relationship between C3a and the other covariates taken together ($F=2.68$, $p=0.006$, $R^2=0.19$), however CST was not a significant predictor ($p=0.65$). The only significant covariate identified was age ($\beta=75.83$, $p=0.02$).

Univariate associations were also explored between reproductive tract infection and other covariates (e.g. age, education level). There were statistically significant associations between age and reproductive tract infection <20 weeks ($t=3.05$; $p=0.003$). Women diagnosed with a reproductive tract infection less than 20 weeks were likely to be younger than the rest of the cohort. There was also a statistically significant association

between infection < 20 weeks and insurance status ($\chi^2$=12.93; p=0.000) as well as marijuana use ($\chi^2$=5.19; p=0.02); women with vaginal infection<20 weeks were more commonly Medicaid insured and more likely to use marijuana.

In the final linear regression analyses, there were no significant relationships identified between reproductive tract infection and Bb (F=1.52, p=0.18, $R^2$=0.11). There was a significant linear relationship identified in the model of reproductive tract infection and C3a (F=3.72, p=0.001, $R^2$ =0.17), however reproductive tract infection was not a significant variable (p=0.51). Age (β=80.90, p=0.006) was the only significant covariate in the model.

In summary, there are no significant relationships identified between complement levels, CSTs, or reproductive tract infection. Age appears to be an important factor as it relates to complement C3a levels. Findings in our study show that as age increases complement C3a levels increase. C3a is a potent inflammatory mediator and marker commonly associated with the classical complement pathway, which involves the more traditional immune response associated with antigen/antibody binding. Women who are older may demonstrate a more robust classical immune response as evidenced by the higher levels of the C3a marker for every one year increase in age; this response could be adaptive and may be related to the lower levels of reproductive tract infections in older women. This finding may also indicate that there are age related changes and potential weathering in the activities of the complement system that promote a heightened inflammatory state. Age associated changes in immune and inflammatory responses have been previously reported in African American women,[15] suggesting that aging may be

associated with dysregulation in immune and inflammatory responses resulting in increased inflammation.

**Chapter 4**

The final manuscript (chapter 4) addresses both the second aim and exploratory aim of this dissertation study. This manuscript summarizes the contribution of complement activation to the development of adverse pregnancy outcomes in a high-risk population by exploring whether higher concentrations of 1$^{st}$ trimester complement activation fragments C3a and Bb are associated with early pregnancy clinical risk factors for PTB (e.g. twenty-week cervical length, health behaviors) as well as the gestational age at delivery. As described earlier, the two previous studies of complement activation during early pregnancy in humans, found that women with elevated complement levels in the highest quartile had an increased risk of developing adverse pregnancy outcomes including PTB.[16-18] However, neither of the studies identified risk factors for elevated complement levels, and as mentioned previously, neither study was completed in a population of women at high risk for adverse outcomes. The dissertation study hypotheses for Aim 2 were that: (1) women with higher concentrations of complement plasma levels of C3a and Bb would have shorter cervical lengths at 20 weeks and/or a shorter gestational age at delivery, and (2) that women with health behaviors previously associated with PTB would demonstrate higher levels of C3a or Bb.

Multivariable linear regression was used to test whether complement factors C3a and/or Bb were significantly associated with the twenty-week cervical length measurement. The results of the regression analyses indicated that only one variable, history of PTB, explained 19% of the variance ($F=3.65$, $p=0.003$, $R^2=0.19$) in the cervical

length measurement. The cervical length would be expected to be 0.59cm higher in woman with no previous history of PTB (β=0.59, p=0.007); neither C3a (β=0.00; p=0.41) or Bb (β=0.40; p=0.23) were significantly associated with cervical length.

Multivariable linear regression also was used to test whether complement factors c3a and Bb were significantly associated with the gestational age at delivery. There was a relationship identified between gestational age and the covariates taken together (F=2.42, p=0.02, $R^2$=0.11), but C3a (p=0.21) and Bb (p=0.54) were not significant variables in the model; Age (β=0.21, p=0.03) and no history of reproductive tract infection <20 weeks (β=-1.66 p=0.04) were the only significant variables. For every one year increase in age, the gestational age would be expected to increase by 0.2 weeks/1.4 days. For a woman without a history of early pregnancy reproductive tract infection, it would be expected that the gestational age at delivery would be 1.6 weeks lower than someone with a history of early pregnancy reproductive tract infection.

An additional linear regression analysis was done to explore factors associated with gestational age among women who had a spontaneous labor only, excluding those who were induced or who presented with no labor. There was not a significant linear relationship identified between gestational age and the covariates taken together (F=1.26, p=0.28; $R^2$=0.11), although a history of PTB was significant variable in the model (β=2.84, p=0.03). Women with no previous history of PTB would be expected to have a gestational age at delivery 2.8 weeks longer than someone with a history of PTB.

A variety of health behaviors associated with either infection or preterm birth were explored in this study. Randomized controlled trials targeting the elimination of risk factors such as infection have not been effective in decreasing the incidence of PTB.[19]

Sexual and vaginal hygiene practices (e.g. douching, multiple sex partners), although not previously shown to have a direct association with an increased PTB risk, may potentially interrupt vaginal protective mechanisms (e.g. ph, lactobacilli, hydrogen peroxide), thereby promoting the growth of microbes that could lead to infection.[20] Sexual health behaviors may also promote changes that alter the integrity of the vaginal and rectal tissues, making the opportunity for infection more favorable. Independent sample t-tests were used to explore differences in the mean concentration of C3a and Bb for select health behaviors (e.g. smoking, marijuana use, sexual and vaginal health behaviors); there were no significant differences in the mean concentration of C3a or Bb identified in the sample.

In summary, C3a and Bb are not associated with the twenty-week cervical length, gestational age at delivery, or various health behaviors in AA women. The most significant factor associated with shorter cervical length was having a history of PTB. Similarly, the most significant factors associated with gestational age in this population were age and a history of PTB. There was an unexpected relationship between early pregnancy infection and gestational age, however, in that women who did not have a reproductive tract infection prior to 20 weeks would have a gestational age at delivery 1.6 weeks less than someone who had an early pregnancy infection. This finding is opposite of what is found in the literature, which suggests that reproductive tract infection is a risk factor for PTB, particularly among AA women.[11] This relationship was further explored using a student t-test to see if there was difference in the mean gestational age between women with and without infection. There were not significant differences in the mean gestational age between the groups ($t=-1.40$, $p=0.16$). The reproductive tract infection

variable in the data set is a variable that takes into account several different types of reproductive tract infections; it may be that teasing out each type of infection and looking at the individual relationships with gestational age would be more meaningful and reflective of findings in the literature. It might also be that women diagnosed with a reproductive tract infection early in pregnancy were more closely monitored for a subsequent infection compared to women not identified with an infection previously; this could have led to consideration of the pregnancy as high risk by providers, leading to closer surveillance of infection and appropriate treatment as pregnancy progressed.

## Discussion

Collectively, these dissertation study findings suggest that the vaginal microbiome composition and reproductive tract infection are not associated with early pregnancy C3a and Bb levels among AA women. Further, C3a and Bb levels are not associated with the twenty-week cervical length or the gestational age at delivery in this sample. The findings of this study suggest that the activities of the complement system are different among AA women than among the samples comprised predominantly of Caucasian women in other studies and do not appear to increase the risk for PTB above the levels observed in this sample (13.9%).

The process of complement initiation and amplification is highly complex and involves complex interactions between several complement effector mechanisms that work together to regulate a wide range of physiologic activities including host defense against bacteria, tissue regeneration and angiogenesis, and embryo implantation.[21] There are many triggers that can tip the scales from normal physiologic function into self attack,

thus the system is held tightly in check by a number of complement regulatory proteins present in the plasma or on host cell membranes.[7] The problems that occur related to complement activation often depend on a balancing act between mechanisms of activation and regulatory mechanism that are designed to prevent uncontrolled complement system activity. Complement regulator proteins are found in various tissues as well as in the circulation. If there is ineffective control of complement activation by the regulatory proteins, complement activation can become excessive and result in host tissue damage and disease.[22,23] It is possible that African American women in this study have efficient complement regulatory proteins that are effective at preventing uncontrolled complement activation and as a result do not have excessively high plasma levels of complement activation fragments (e.g. C3a and Bb). Despite this possibility, there is a relatively high rate of PTB in this cohort (13.9%), the findings of this study suggest that systemic markers of complement activation do not further increase the risk for adverse outcomes.

The previous complement studies were conducted in primarily white populations, and it possible that the underlying pathophysiology associated with complement system in these populations may also be due to differences in the activities of regulatory proteins. To better understand this process, future studies may benefit from exploring a more comprehensive measure of complement system activity that includes measures of not only complement activation fragments, but also other effector mechanisms of the complement pathway including other anaphylatoxins (ex C5a), opsonins (C3b, C3d), complement regulatory proteins (e.g. Factor H, CR1) and markers of the membrane attack complex (C5b-C9). Effective interplay between these factors ensures effective

complement system function, which results in an effect defense against bacteria and well controlled inflammation due to tight control of inflammatory mediators.

The study also found that the vaginal microbiome CSTs were not associated with the complement factors. It is well established that pathogens have the ability to mutate and evolve mechanisms that prevent their effective elimination by various immune mechanisms. Pathogens have been shown previously to effectively dampen complement activation and evade elimination by affecting factors that regulate activation, amplification, phagocytosis, and cell lysis.[24] Bacteria are able to accomplish this via the creation of a variety of mechanisms, such as microbial proteases, that stimulate proteolysis resulting in the effective breakdown of complement proteins. For example, if C3a were the targeted complement protein there would be reduced recruitment of inflammatory cells and localized inflammation thus preventing effective elimination of the target pathogen. Many women in this sample had vaginal microbiome CSTs that were not comprised of the traditional healthy type lactobacillus, in fact many of the microbiome CSTs were dominated by traditionally pathogenic genera of bacteria (e.g. *Prevotella, Snethia, Shuttleworthia*). Markers of complement activation may actually be blunted in African American women as a consequence of persistent exposure to pathogenic microbes that have evaded elimination via the dampening of complement activation mechanisms.

Age appears to be an important factor in this study as it was significantly associated with C3a levels, reproductive tract infection <20 weeks, and the gestational age at delivery. Specifically, it was found in Aim 1 that age was a significant predictor of C3a concentration ($\beta$=75.83, p=0.02). There was also a statistically significant

association between age and reproductive tract infection <20 weeks (t=3.05; p=0.003). Women diagnosed with a reproductive tract infection less than 20 weeks were likely to be younger than the rest of the cohort. In aim 2, it was shown that age was a significant predictor of gestational age (β=0.21, p=0.03). As previously discussed, women who are older may demonstrate a more robust classical immune response as evidenced by the higher levels of the C3a marker for every one year increase in age; this response could be adaptive and may be related to the lower levels of reproductive tract infections in older women. This finding may also indicate that there are age related changes and potential weathering in the activities of the complement system that promote a heightened inflammatory state. Age associated changes in immune and inflammatory responses have been previously reported in African American women,[15] suggesting that aging may be associated with dysregulation in immune and inflammatory responses resulting in increased inflammation.

In conclusion, the finding that C3a and Bb do not associate with select outcomes, does not mean that the complement system is of no importance among AA women during pregnancy. There are many factors that influence complement system physiology that may need to be explored further in AA women to determine how this system influences pregnancy outcomes.

## Strengths and Limitations

This study was the first to examine a well-supported innate immune mechanism that has been understudied in the context of PTB—complement activation--in a population with the highest incidence of PTB, AA women. Previous complement studies have demonstrated that elevated levels of early pregnancy complement activation factors

C3a and Bb are associated with PTB, however these studies were in relatively homogenous populations with only 5-7% of the sample represented by AA women. This study was also unique in that it employed a design consistent with recommended frameworks for studying racial disparities which recognize that a requisite first step in studying disparities is to understand the interplay of factors within the disparate group.[25] To our knowledge, this is the first study of complement activation in an exclusively AA population. Although the hypotheses in this study were not supported, the findings from this study provides a significant contribution to the literature on the role of complement system and the relationship to select pregnancy outcomes among AA women during pregnancy.

There were several limitations identified in this dissertation research study. First, there were issues with the quality of C3a measurement due to the high interassay (plate to plate variability) for C3a. To address this issue, a variable called "assay plate" was created and controlled for in the final regression model, however the initial regression analyses found that this variable explained most of the variance in the C3a concentration. To reduce the influence of the assay plate variable, C3a analyses were repeated using only women with less than 10% variation in concentration between the sample duplicates. While improving the rigor of the bioassay results, this led to a decrease in subject number, affecting the ability to detect significant findings. Although the assays were completed via collaboration between an experienced lab expert and the doctoral candidate, the limited experience of the student in the laboratory may have influenced the results. The quality measurement of biomarkers is critical to sound science, and the literature supports that one factor that affects the quality of biomarker measurement is the

experience of the person performing the assays.[26] Secondly, the complement system is highly complex with many influencing factors that vary from person to person. In this study the biomarkers were measured from the systemic circulation and compared to the vaginal microbiome, a more localized environment. It may have been more beneficial to look at local complement activity along with other markers of inflammation in the vaginal and cervical environment to understand more about the local inflammatory profile specific to the vaginal microbiome. The C3a and Bb levels present in the systemic circulation may be more associated with factors not measured in this study including the presence of infection at other body sites or the influence from microbes present at other microbiome sites.

It was also found that the complement biomarkers were not associated with the select pregnancy outcomes. This finding may be a result of the strict nature of the larger study inclusion/exclusion criteria which may have eliminated the women who are more likely to have adverse pregnancy outcomes and/or excessive complement activation, such as women with chronic medical conditions or other risk factors for exaggerated inflammation. The challenges of including high risk pregnant women with chronic conditions in research studies are significant as pregnant women represent a vulnerable population; nevertheless, these strict criteria may influence the ability to detect mechanisms associated with adverse outcomes simply because the women enrolled into the study may not be representative of the populations at highest risk for poor outcomes.

## Implications for Future Research

While this study may not have found significant associations between the complement factors C3a and Bb and pregnancy outcomes, this work does suggest that more research into other aspects of the complement system may be important to investigate in AA women. The activation and amplification of the complement system is tightly regulated to avoid excessive activation and exaggerated inflammation, however ineffective activation could also set the stage for a dysfunctional inflammatory response, which could also have consequences during pregnancy. Other studies have shown that pathogens have developed ways to avoid elimination by the complement system via the creation of mechanisms including microbial proteases and complement evasion proteins. More studies are needed to confirm how this system functions in pregnant AA women, as the activities of the complement system are very complex and the measurement of one or two markers of inflammation is likely not sufficient to rule in or out the complement system as a contributor to adverse outcomes. The next steps to move this research topic forward are outlined in the figure below (Figure 1):

| **Next Steps** |
| --- |
| Consider a more comprehensive evaluation of complement system activity (e.g. markers of activation, complement receptors, regulatory proteins) within the vaginal environment along with other local markers of inflammation and the relationship to the vaginal microbiome CSTs |

| |
|---|
| Explore how various markers of complement system activity function within various vaginal microbiome community state types; explore the relationships with different pregnancy outcomes. |
| Consider exploring the vaginal microbiota for markers of complement evasion (e.g. extracellular fibrinogen binding protein which targets the C3 convertase) in AA women. |
| Investigate the role of C3a and the relationship to the microbiome and various pregnancy outcomes at different age points. |

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